Biochimica et Biophysica Acta 850 (1986) 146–155
Elsevier

BBA 42026

Partial degradation of the 18-kDa protein of the photosynthetic oxygen-evolving complex: a study of a binding site

Tomohiko Kuwabara [a], Teruyo Murata [a], Mitsue Miyao [b] and Norio Murata [b,*]

[a] *Department of Chemistry, Faculty of Science, Toho University, Miyama, Funabashi 274, and* [b] *National Institute for Basic Biology, Myodaiji, Okazaki 444 (Japan)*

(Received January 27th, 1986)

Key words: Chloride effect; Oxygen evolution; Thylakoid protein; Proteinase; (Spinach)

When the NaCl extract from spinach Photosystem II particles was dialyzed against a low-salt medium, the 18-kDa protein slowly degraded to a fragment of 17 kDa. This observation suggests that a proteinase previously associated with the Photosystem II particles in a latent form was activated by dissociation with NaCl. The 18-kDa protein and the 17-kDa fragment were purified, and their N-terminal amino acid sequences and total amino acid compositions were determined. These results determined 44 amino acid residues at the N-terminal of the 18-kDa protein, and suggest that 12 amino acid residues (mostly hydrophobic) at the N-terminal were lost by the degradation. The 18-kDa protein could rebind to the NaCl-treated and 24-kDa protein-supplemented Photosystem II particles and sustain their oxygen-evolution activity in a low-Cl^- medium, whereas the 17-kDa fragment had lost these abilities. These observations suggest that the N-terminal region of the 18-kDa protein forms a domain which binds to Photosystem II particles.

Introduction

Biochemical studies on photosynthetic oxygen evolution have revealed that extrinsic proteins having molecular masses of 33 kDa, 24 kDa and 18 kDa are components of the oxygen-evolving complex [1–3]. A stoichiometric determination indicates that the oxygen-evolving complex contains one molecule each of the three extrinsic proteins per photochemical reaction center II [4]. Among these proteins, the 33-kDa protein was the first to be isolated and characterized chemically and physicochemically [5,6]. Jansson et al. [7] purified and characterized the 24-kDa protein from an acetone powder of spinach thylakoids. Partial purification and characterization of the 18-kDa protein have also been reported [8,9]. However, this protein is degraded to a slightly smaller fragment of 17 kDa during purification, possibly by a latent proteinase associated with the thylakoid membranes (see Results). Therefore, characterization of this degradation is a prerequisite for the purification and further characterization of this protein.

The 18-kDa protein is known to preserve oxygen-evolution activity of PS II particles suspended in a low-Cl^- medium [10,11], although the detailed mechanism of this process is still in question. The 18-kDa protein seems to bind specifi-

* To whom correspondence should be addressed.

Abbreviations: Chl, chlorophyll; PS, Photosystem; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; SDS, sodium dodecyl sulphate.

cally to the 24-kDa protein in PS II particles, since it only binds with low affinity to PS II particles lacking the 24-kDa protein [12].

In the present study, the 18-kDa protein and its 17-kDa fragment were purified and characterized. By comparing their molecular and functional properties, the structure of the binding site of the 18-kDa protein to the oxygen-evolving complex is suggested.

Materials and Methods

Preparation of PS II particles

Spinach was purchased from a local market. Chloroplasts were isolated with 200 mM NaCl/100 mM sucrose/50 mM sodium phosphate–potassium phosphate (pH 7.4) as described previously [1]. PS II particles were prepared from the chloroplasts with Triton X-100 as described previously [1] except that the chloroplasts were suspended in 100 mM NaCl/300 mM sucrose/50 mM sodium phosphate–potassium phosphate (pH 6.4) instead of 50 mM NaCl/300 mM sucrose/50 mM sodium phosphate–potassium phosphate (pH 6.9) for Triton X-100 treatment. The increase in NaCl concentration and decrease in pH value provided a higher yield of PS II particles; about 40% of the Chl in the chloroplasts was routinely recovered in prepared PS II particles. The isolated PS II particles were used immediately for experiments, or suspended in 30% (v/v) ethylene glycol/10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) and kept frozen at 77 K until use.

Purification procedure

After thawing, PS II particles corresponding to 140 mg Chl were washed twice with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) by suspension and centrifugation. To extract the 18-kDa protein together with the 24-kDa and 33-kDa proteins, the washed particles were suspended in 70 ml of 1 M $CaCl_2$/25 mM Mes-NaOH (pH 6.5) at a Chl concentration of about 2 mg · ml^{-1} and incubated for 20 min under room light [13]. The suspension was centrifuged at 35 000 × *g* for 20 min. The supernatant was incubated at 0°C for 3 h in order to degrade proteinase activities (see Results). The extract was dialyzed against 5 mM Mes-NaOH (pH 6.5) twice, then 300 mM sucrose/5 mM Mes-NaOH (pH 6.5), and finally 300 mM sucrose/10 mM sodium phosphate (pH 6.6). Each step of dialysis was carried out using 30 vol. of the dialysis medium for about 6 h. The dialyzate was centrifuged at 200 000 × *g* for 30 min. The supernatant was applied to a DEAE-Sepharose CL-6B column (2.6 cm i.d. × 10 cm) which had been equilibrated with 10 mM sodium phosphate (pH 6.6). The 18-kDa protein was not adsorbed and was thoroughly eluted out with 130 ml of 20 mM sodium phosphate (pH 6.5). The 24-kDa protein was eluted with 160 ml of 40 mM NaCl/20 mM sodium phosphate (pH 6.5). The 33-kDa protein was eluted, following washing of the column with 60 ml of 50 mM NaCl/20 mM sodium phosphate (pH 6.5), with a gradient from 50 to 150 mM NaCl in 20 mM sodium phosphate (pH 6.5). For further purification, the 18-kDa protein fraction was applied to a CM-Toyopearl 650 M column (1.6 cm i.d. × 10 cm) which had been equilibrated with 20 mM sodium phosphate (pH 6.6). The column was washed with 60 ml of 40 mM NaCl/20 mM sodium phosphate (pH 6.5). The 18-kDa protein was eluted with 100 ml of 50 mM NaCl/20 mM sodium phosphate (pH 6.5). The 24-kDa protein fraction was concentrated to about 20 ml with a Diaflo membrane (YM-5, Amicon), dialyzed against 50 vol. of 10 mM sodium phosphate (pH 6.6) for 6 h and applied to a CM-Sepharose CL-6B column (1.6 cm i.d. × 10 cm) which had been equilibrated with 10 mM sodium phosphate (pH 6.5). After the column had been washed with 60 ml of 20 mM sodium phosphate (pH 6.5), the 24-kDa protein was eluted with 130 ml of 30 mM NaCl/20 mM sodium phosphate (pH 6.5).

For purification of the 17-kDa fragment of the 18-kDa protein, PS II particles washed with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) were suspended in 1 M NaCl/25 mM Mes-NaOH (pH 6.5) at a Chl concentration of 2 mg · ml^{-1}. After standing for 20 min, the suspension was centrifuged at 35 000 × *g* for 20 min, and the supernatant containing the 18-kDa and 24-kDa proteins was dialyzed against 30 vol. of 10 mM sodium phosphate (pH 6.6) at 7°C for 72 h, renewing the dialysis medium every day. This dialysis procedure degraded the 18-kDa protein to a slightly smaller fragment of 17 kDa (see Results).

The dialyzate was centrifuged at 200000 × *g* for 30 min. The 17-kDa fragment was purified from the supernatant by column chromatography with DEAE-Sepharose CL-6B and CM-Toyopearl 650 M as conducted in the purification of the 18-kDa protein except that the 17-kDa fragment was eluted from the CM-Toyopearl column with a 50–150 mM NaCl gradient in the presence of 20 mM sodium phosphate (pH 6.5).

Analytical methods

SDS-urea-polyacrylamide gel electrophoresis was carried out using the buffer system of Laemmli [14] in the presence of 6 M urea in both stacking and resolving gels. Samples were dissolved in 2% SDS/10% glycerol/3 mM dithiothreitol/62.5 mM Tris-HCl (pH 6.8). The acrylamide concentrations of the stacking and resolving gels were 5% and 15%, respectively. Gel plates were stained with Coomassie brilliant blue R-250 [5]. Densitometric traces of the stained gel plates were obtained using a dual-wavelength TLC scanner (CS-910, Shimadzu) as described previously [5]. Relative molecular masses of the proteins and the fragments were estimated by SDS-polyacrylamide gel electrophoresis, which was performed as above but in the absence of urea. The standards used for molecular mass estimation were bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

The N-terminal amino acid sequences were determined by the Edman degradation method [15] in which amino acid phenylthiohydantoins were identified with protein sequence analyzers (Applied Biosystem, 470A, and Jeol, JAS-47K) according to Zimmerman et al. [16]. For amino acid analysis, samples were hydrolyzed in 6 M HCl at 110°C for 24 h, and applied to an amino acid analyzer (Hitachi 835). Cysteine was determined by hydrolyzing the sample after oxidation with performic acid [17]. Tryptophan was determined from the absorption spectrum of the protein in 0.1 M NaOH according to Goodwin and Morton [18].

The effective molecular size of the protein in the absence of SDS was determined by HPLC in the gel filtration mode using a series of two columns of TSK-GEL G 3000 SW (Toyosoda, 0.75 cm i.d. × 60 cm each) with an elution medium of 250 mM NaCl/25 mM Mes-NaOH (pH 6.5) as described previously [19]. The standards used for molecular size estimation were bovine serum albumin (67 kDa), ovalbumin (43 kDa), trypsin inhibitor (20.1 kDa) and RNAase A (13.7 kDa).

Isoelectric focusing was carried out in a polyacrylamide gel using a flat-bed apparatus (FBE 3000, Pharmacia). The gel mixture contained 6.7% acrylamide/0.32% *N,N'*-methylenebisacrylamide/2.1% Ampholine (a mixture of pH 9–11 and pH 3.5–10, 4:1)/10% glycerol/0.0005% riboflavin/0.056% *N,N,N',N'*-tetramethylethylenediamine/0.017% ammonium peroxydisulphate. Polymerization was initiated by exposing the gel mixture to light from a fluorescent lamp. Solutions of anode and cathode electrodes were 10 mM H_3PO_4 and 1 M NaOH, respectively. The pH gradient of the gel plate was measured at 4°C on the gel surface with a surface electrode (6210-06T, Horiba) at a 1-cm interval. The gel plate was stained according to Otavsky and Drysdale [20] using Coomassie brilliant blue R-250 and $CuSO_4$.

Rebinding of the 18-kDa protein and 17-kDa fragment to NaCl-treated PS II particles

PS II particles were treated with 1 M NaCl/25 mM Mes-NaOH (pH 6.5) at a Chl concentration of 0.3 mg · ml^{-1}. The treated particles were washed with, and suspended in, 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). Then they were incubated at 0°C for 30 min with either the 24-kDa protein alone, the 24-kDa protein plus the 18-kDa protein, or the 24-kDa protein plus the 17-kDa fragment at the designated protein-to-Chl ratio. These (partially) reconstituted PS II particles were used to study the time-dependent inactivation of oxygen evolution in a low-Cl^- medium and the rebinding ability of the 18-kDa protein and 17-kDa fragment.

Others

Oxygen-evolution activity was measured at 25°C with a Clark-type oxygen electrode in the presence of 0.3 mM phenyl-*p*-benzoquinone and 0.05% bovine serum albumin [1]. Chl concentration was determined in 80% acetone [21]. Concentrations of the 24-kDa and 18-kDa proteins were determined using their absorption coeffi-

cients at 277 nm of 26 and 13 $mM^{-1} \cdot cm^{-1}$, respectively [8]. The absorption coefficient of the 17-kDa fragment was assumed to be the same as that of the 18-kDa protein.

Results

Proteinase activity associated with PS II particles in a latent form

Treatment of PS II particles with 1 M NaCl (pH 6.5) released the 18-kDa and 24-kDa proteins but not the 33-kDa protein [19]. One might expect that such an extraction method would be advantageous for the purification of the 18-kDa and 24-kDa proteins, since the extract contains only these two proteins as main components [19]. However, when the extract was dialyzed at 7°C against 20 mM sodium phosphate (pH 6.6), which was a necessary step before ion-exchange chromatography, new bands emerged at 17 kDa and 22 kDa. Fig. 1 shows a time-dependent change in the polypeptide composition of the extract during the dialysis. The 17-kDa band was detected after dialysis for 3 h and then gradually increased with further dialysis. In contrast, the 18-kDa protein band became faint in the course of the dialysis and diminished at 48 h. This result suggests that the 17-kDa polypeptide was a degradation product of the 18-kDa protein. Thus, the polypeptide is hereafter termed '17-kDa fragment' of the 18-kDa protein. The 22-kDa polypeptide may be ascribed also to be a degradation product from the 24-kDa protein.

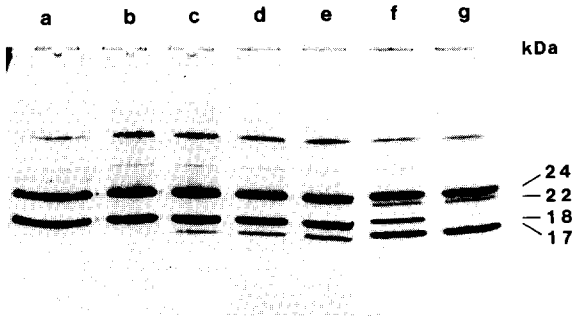

Fig. 1. The change during dialysis in polypeptide composition of NaCl-extract from PS II particles. PS II particles were treated with 1 M NaCl/25 mM Mes-NaOH (pH 6.5), and the extract obtained as the supernatant by centrifugation was dialyzed against 400 vol. of 20 mM sodium phosphate (pH 6.6) for 0 h (a), 1 h (b), 3 h (c), 5 h (d), 7 h (e), 24 h (f) or 48 h (g) at 7°C.

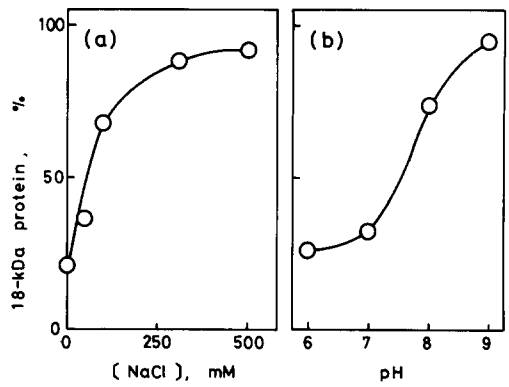

Fig. 2. Effect of NaCl concentration and pH on the degradation of the 18-kDa protein into the 17-kDa fragment during dialysis of NaCl-extract from PS II particles. The extract was dialyzed for 24 h at 7°C (a) against 400 vol. of 20 mM sodium phosphate (pH 6.5) containing various concentrations of NaCl, or (b) against 20 mM buffer, either Mes-NaOH (pH 6.0), Hepes-NaOH (pH 7.0), Tricine-NaOH (pH 8.0) or Tricine-NaOH (pH 9.0), and then subjected to SDS-urea-polyacrylamide gel electrophoresis. Relative amounts of 18-kDa protein and 17-kDa fragment were estimated from peak heights of the densitometric tracing of the gel. The ordinate indicates the amount of 18-kDa protein relative to the sum of the amounts of the 18-kDa protein and 17-kDa fragment, which were estimated from peak heights of the densitometric tracing of SDS-urea-polyacrylamide gel electrophoresis with correction for relative staining intensities of the two components.

Fig. 2a shows the effect of NaCl concentration during dialysis on the degradation of the 18-kDa protein. The protein degraded at NaCl concentrations below 100 mM, but not above 300 mM. The most plausible explanation for this phenomenon is that the 18-kDa protein was hydrolyzed by a proteinase which was inactive in a high-salt medium but became active in a low-salt one, and that the proteinase was associated with PS II particles, and releasable with 1 M NaCl. The activity of this proteinase may have been latent in the membrane-bound form, since PS II particles did not contain a polypeptide corresponding to the 17-kDa fragment. Fig. 2b shows the effect of the pH of the dialysis medium on the conversion of 18-kDa protein into 17-kDa fragment. This protein degraded at pH 6–7, but was stable at pH 9. The dialysis at pH lower than 6 produced white precipitates.

In order to protect the 18-kDa and 24-kDa proteins from the degradation during dialysis, several proteinase inhibitors were tested. Without effect were 0.1 mM sodium *p*-chloromercuribenzoate, 1 mM *N*-ethylmaleimide, 10 mM sodium iodoacetate, 1 mM dithiothreitol, 2 mM phenylmethylsulphonyl fluoride, 2.5 mM 1,10-phenanthroline, 10 mM EDTA, 5 mM 5-amino-*n*-caproic acid, 1 mM α-*N*-benzoyl-L-arginine and 5 mM benzamidine-HCl. However, once the extract had been dialyzed against 1 M $CaCl_2$ (pH 6.5) or 1 M Tris-HCl (pH 9.3) for 3–24 h, the 18-kDa and 24-kDa proteins did not degrade during subsequent dialysis in a low-salt medium at pH 6.5 (Fig. 3), suggesting that these treatments inactivate the supposed proteinase.

Purification of the 18-kDa protein and the 17-kDa fragment

The 18-kDa protein was extracted from PS II particles with 1 M $CaCl_2$ together with the 24-kDa and 33-kDa proteins [22]. The use of $CaCl_2$ as the extraction medium was advantageous, since it achieved both extraction of the proteins and inactivation of the supposed proteinase. When the extract was dialyzed for 48 h prior to column chromatography, trace amounts of the 17-kDa and 22-kDa fragments and an unknown polypeptide of 15 kDa were produced in addition to the 18-kDa, 24-kDa and 33-kDa proteins. The amounts of 17-kDa and 22-kDa fragments were less than 2% of those of 18-kDa and 24-kDa proteins, respectively.

The 18-kDa, 24-kDa and 33-kDa proteins were successfully separated by anion-exchange column chromatography with DEAE-Sepharose CL-6B (Fig. 4). The resultant 18-kDa protein fraction contained trace amounts of 15-kDa and 17-kDa polypeptides as detectable contaminants. It was then subjected to cation-exchange column chromatography with CM-Toyopearl 650 M. With 50 mM NaCl/20 mM sodium phosphate (pH 6.5), the 18-kDa protein was eluted first and the 17-kDa fragment just after the 18-kDa protein with partial

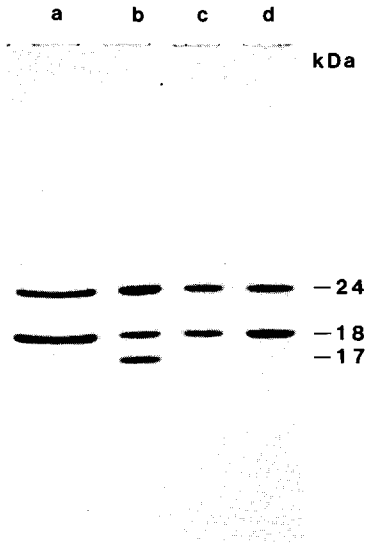

Fig. 3. SDS-urea polyacrylamide gel electrophoresis of NaCl-extract from PS II particles. (a) Before dialysis, (b) dialyzed against 20 mM sodium phosphate (pH 6.6), (c) pre-dialyzed against 1 M $CaCl_2$/25 mM Mes-NaOH (pH 6.5) and subsequently dialyzed against 5 mM Mes-NaOH (pH 6.5), or (d) predialyzed against 1 M Tris-HCl (pH 9.3) and subsequently dialyzed against 200 mM sodium phosphate (pH 6.4) and then 20 mM sodium phosphate (pH 6.6). In (b)–(d), dialysis and predialysis were carried out in 400 vol. of the dialysis medium for 24 h at 7°C.

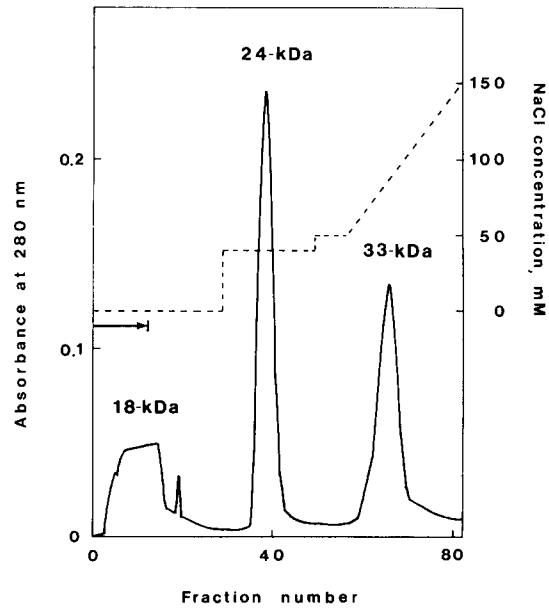

Fig. 4. Separation of the 18-kDa, 24-kDa and 33-kDa proteins in $CaCl_2$ extract from PS II particles by column chromatography with DEAE-Sepharose CL-6B. The chromatography was performed at 10°C at a flow rate of 100 $ml \cdot h^{-1}$. Fraction volume was 8 ml. Loading of the sample solution is indicated by the arrow at the left.

overlapping. The purity of the resultant 18-kDa protein preparation was estimated to be 99.5% by SDS-urea polyacrylamide gel electrophoresis. By this method, 7 mg of the 18-kDa protein were obtained from PS II particles corresponding to 140 mg Chl. The 24-kDa protein fraction from the DEAE-Sepharose CL-6B column was further purified by column chromatography with CM-Sepharose CL-6B. The 33-kDa protein was purified only with the DEAE-Sepharose CL-6B column. These protein fractions showed no contaminant in SDS-urea polyacrylamide gel electrophoresis.

For purification of the 17-kDa fragment, the extract from PS II particles with 1 M NaCl was dialyzed against 10 mM sodium phosphate (pH 6.6) for 3 days, during which the 18-kDa protein completely degraded to the 17-kDa fragment. Then, the 17-kDa fragment was purified from the dialyzate by column chromatography with DEAE-Sepharose CL-6B and CM-Toyopearl 650 M. The resultant 17-kDa fragment preparation did not contain any contaminant detectable by SDS-urea-polyacrylamide gel electrophoresis.

N-terminal amino acid sequences and physicochemical characteristics of the 18-kDa protein and the 17-kDa fragment

The N-terminal amino acid sequences of the 18-kDa protein and 17-kDa fragment are presented in Table I. The sequence from the 13th to the 26th amino acid residues of the 18-kDa protein was the same as that from the 1st to the 14th in the 17-kDa fragment. This suggests that the degradation of the 18-kDa protein to the 17-kDa fragment during the dialysis of the NaCl extract should be ascribed to the removal of 12 amino acid residues at the N-terminal region. This sequence was very hydrophobic; it contained ten hydrophobic amino acids and only two hydrophilic ones (glutamic acid and arginine, which were located at the N-terminal and the third residue, respectively). It was unique also in containing five proline residues, four of which were in

TABLE I

THE N-TERMINAL AMINO ACID SEQUENCES OF THE 18-kDa PROTEIN AND ITS 17-kDa FRAGMENT

18 kDa		17 kDa		18 kDa		17 kDa	
No.	amino acid	No.	amino acid	No.	amino acid	No.	amino acid
1	Glu			23	Ser	11	Ser
2	Ala			24	Asp	12	Asp
3	Arg			25	Gln	13	Gln
4	Pro			26	Ala	14	Ala
5	Ile			27	–	15	Arg
6	Val			28	Asp	16	Asp
7	Val			29	Gly	17	Gly
8	Gly			30	–	18	Thr
9	Pro			31	Leu.	19	Leu
10	Pro			32	–	20	Pro
11	Pro			33	–	21	Tyr
12	Pro			34	–	22	Thr
13	Leu	1	Leu	35	–	23	Lys
14	Ser	2	Ser	36	–	24	Asp
15	Gly	3	Gly	37	–	25	Arg
16	Gly	4	Gly	38	–	26	Phe
17	Leu	5	Leu	39	–	27	Tyr
18	Pro	6	Pro	40	–	28	Leu
19	Gly	7	Gly	41	–	29	Gln
20	Thr	8	Thr	42	–	30	Pro
21	Glu	9	Glu	43	–	31	Leu
22	Asn	10	Asn	44	–	32	Pro

TABLE II

AMINO ACID COMPOSITIONS OF THE 18-kDa PROTEIN AND ITS 17-kDa FRAGMENT

Amino acid	Mol%		Residue/protein (mol/mol) [a]			Amino acids removed [b]
	18 kDa	17 kDa	18 kDa	17 kDa	difference	
Lys	9.3	10.1	14.4	14.4	0.0	0
His	1.0	1.3	1.5	1.9	−0.4	0
Arg	5.8	5.4	8.9	7.7	+1.2	1
Asx	10.5	11.3	16.2	16.2	0.0	0
Thr	5.2	5.6	8.0	8.0	0.0	0
Ser	7.8	8.4	12.1	12.0	+0.1	0
Glx	10.9	11.2	16.9	16.0	+0.9	1
Pro	9.1	6.2	14.1	8.9	+5.2	5
Gly	4.9	4.6	7.6	6.6	+1.0	1
Ala	8.9	9.0	13.8	12.9	+0.9	1
Cys/2	0.0	–	0.0	–	–	0
Val	4.5	3.7	7.0	5.3	+1.7	2
Met	0.0	0.0	0.0	0.0	0.0	0
Ile	4.6	4.4	7.1	6.3	+0.8	1
Leu	11.7	12.7	18.1	18.2	−0.1	0
Tyr	3.4	3.4	5.2	4.9	+0.3	0
Phe	2.0	2.2	3.1	3.1	0.0	0
Trp	0.6	0.5	0.9	0.7	+0.2	0

[a] Values were obtained assuming the molecular weight of 18-kDa protein and 17-kDa fragment to be 18000 and 16800, respectively.
[b] Determined from the partial amino acid sequences.

series, and in the hydrolysis by the proteinase occurring at the carboxy end of this series of proline residues.

Amino acid analysis (Table II) revealed that both the 18-kDa protein and the 17-kDa fragment were rich in polar amino acids such as aspartic acid/asparagine, and glutamic acid/glutamine, and also leucine, but devoid of the sulphur-containing amino acids, methionine and cysteine. On assumption that the 17-kDa fragment was shorter by the 12 amino acid residues, which corresponds to a molecular mass of 1.2 kDa less than that of the native protein, the amino acid eliminated with the degradation could be calculated from the amino acid compositions of the 18-kDa protein and 17-kDa fragment. The result (Table II, column 6) was very consistent with that obtained by the analysis of the partical amino acid sequences.

The molecular characteristics of the 18-kDa protein and 17-kDa fragment are summarized in Table III. The molecular mass of the 18-kDa protein was estimated to be 18 kDa by SDS-polyacrylamide gel electrophoresis, but the protein was eluted at the molecular mass region of 23 kDa on gel filtration chromatography in 250 mM NaCl but without SDS. This observation may suggest that the molecular shape of the 18-kDa protein is not a sphere, which would make its effective size much larger than expected from the molecular mass. The molecular mass of the 17-kDa fragment was estimated to be 17 kDa and 18 kDa by SDS-polyacrylamide gel electrophoresis and gel

TABLE III

PHYSICOCHEMICAL CHARACTERISTICS OF THE 18-kDa PROTEIN AND ITS 17-kDa FRAGMENT

PAGE, polyacrylamide gel electrophoresis.

Protein	Molecular mass (kDa)		pI [a]	λ^{b}_{max} (nm)	ϵ at λ^{c}_{max} ($M^{-1} \cdot cm^{-1}$)
	SDS-PAGE	HPLC			
18 kDa	18	23	9.5	277	13000
17 kDa	17	18	9.6	277	not determined

[a] Isoelectric point.
[b] Absorption maximum.
[c] Absorption coefficient at absorption maximum.

filtration chromatography without SDS, respectively. The finding that the 18-kDa protein and 17-kDa fragment differ greatly in effective molecular size as estimated by gel filtration chromatography irrespective of the small molecular mass change may suggest that the molecular shape was considerably altered by the conversion of 18-kDa protein into 17-kDa fragment. The isoelectric points of 18-kDa protein and the 17-kDa fragment were about the same (Table III). Both protein and fragment showed the same absorption spectra, with maximum at 277 nm but no absorption in the visible region (data not shown; see Ref. 8 for the absorption spectrum of the 18-kDa protein).

Function of the 18-kDa protein and the 17-kDa fragment

The 18-kDa protein functions cooperatively with the 24-kDa protein in the oxygen-evolving complex as a Cl^- concentrator [10,11]. Whether the 17-kDa fragment retains the functional property of the 18-kDa protein was studied by comparing NaCl-treated PS II particles supplemented with stoichiometric amounts of 24-kDa protein together with 18-kDa protein or 17-kDa fragment in terms of time-dependent inactivation of oxygen evolution in a low-Cl^- medium (Fig. 5). The activity of the particles supplemented with the 24-kDa protein alone declined in a biphasic manner during the incubation at a low Cl^- concentration; rapid inactivation occurred within 3 min and a slow phase followed. As previously observed [11], the 18-kDa protein eliminated the rapid inactivation at the low Cl^- concentration of the NaCl-treated and 24-kDa protein-supplemented PS II particles, evidence for the function of 18-kDa protein as a Cl^- concentrator. The 17-kDa fragment, on the other hand, did not show such an effect, indicating that the 17-kDa fragment had lost the functional property of the 18-kDa protein.

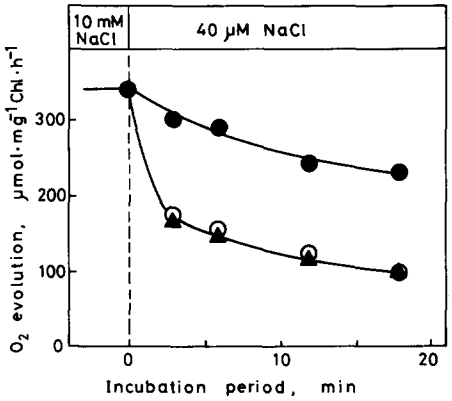

Fig. 5. Effect of the 18-kDa protein and 17-kDa fragment on the time-dependent inactivation of the oxygen evolution of PS II particles in a low-Cl^- medium. The 18-kDa protein or 17-kDa fragment was added to NaCl-treated and 24-kDa protein-supplemented PS II particles in 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) at a Chl concentration of 2.5 mg·ml^{-1}. The protein-to-Chl ratios were 0.24, 0.18 and 0.17 (w/w) for the 24-kDa protein, 18-kDa protein and 17-kDa fragment, respectively. The suspension was diluted 250-fold with 300 mM sucrose/25 mM Mes-NaOH (pH 6.5) to give Chl and NaCl concentrations of 10 μg·ml^{-1} and 40 μM, respectively, and incubated at room temperature with gentle stirring. A portion of the suspension was withdrawn and its oxygen-evolution activity was measured at the designated time. No further addition, ○; the 18-kDa protein added, ●; the 17-kDa fragment added, ▲.

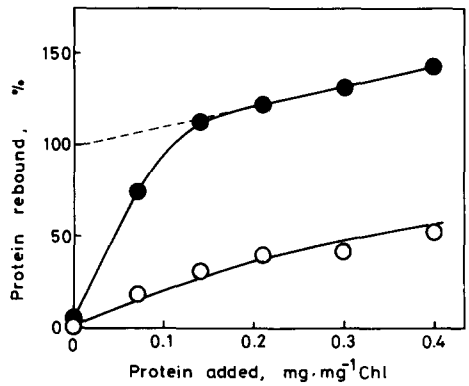

Fig. 6. The rebinding of the 18-kDa protein and 17-kDa fragment to NaCl-treated and 24-kDa protein-supplemented PS II particles. The designated amounts of 18-kDa protein or 17-kDa fragment were added to NaCl-treated and 24-kDa protein-supplemented PS II particles with the added 24-kDa protein-to-Chl ratio of 0.24 (w/w) in 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). After the suspension had been diluted with 5 vol. of the same medium, the particles were collected by centrifugation at 35000 × *g* for 15 min and washed three times with the same medium by resuspension and recentrifugation. Relative amounts of the 18-kDa protein and 17-kDa fragment rebound to PS II particles were estimated from peak heights of the densitometric tracing of SDS-urea-polyacrylamide gel electrophoresis with correction for relative staining intensities of the two components. The 100% level corresponds to the native level of 18-kDa protein in the untreated PS II particles. ●, 18-kDa protein added; ○, 17-kDa fragment added.

The 18-kDa protein and 17-kDa fragment were compared with respect to their capability for rebinding to PS II particles (Fig. 6). When added to NaCl-treated PS II particles supplemented with a stoichiometric amount of the 24-kDa protein, the 18-kDa protein rebound to the particles in a biphasic manner as observed previously [12]; in the high-affinity binding the rebinding was saturated at a low protein-to-Chl ratio, and in the low-affinity binding it was not saturated in the range of protein-to-Chl ratios used in the present experiment. Extrapolation of the straight line in the region of high protein-to-Chl ratios to the ordinate suggests that the saturated level of high-affinity binding corresponded to the native abundance level of the 18-kDa protein in untreated PS II particles. In contrast, the rebinding of the 17-kDa fragment revealed only the unsaturating low-affinity binding. Therefore, the degradation of the 18-kDa protein to 17-kDa fragment resulted in a loss of the high-affinity binding.

When the 18-kDa protein or 17-kDa fragment was added to NaCl-treated PS II particles with no supplementation of the 24-kDa protein, only the low-affinity binding was observed (data not shown). This suggests that the 18-kDa protein specifically binds to the 24-kDa protein with high affinity, whereas both 18-kDa protein and 17-kDa fragment bind to PS II particles with low affinity, regardless of the presence of 24-kDa protein. This low-affinity binding may be due to an electrostatic interaction between negatively charged membrane surface and positively charged protein surface.

Discussion

The present study indicates that the 18-kDa protein degrades to its 17-kDa fragment when it is extracted with 1 M NaCl and dialyzed against a low-salt medium. This degradation seems to be catalyzed by a proteinase which is latent when associated with the PS II particles and becomes active after solubilization with NaCl. The finding that the supposed proteinase was released from the particles with 1 M NaCl may suggest that it is bound to the particles by electrostatic interaction. The 17-kDa fragment did not further degrade during the dialysis within the time range of days. These observations suggest that the substrate specificity of the proteinase is rather strict. It is interesting in this respect that the 18-kDa protein was cleaved after the series of four proline residues. It seems most probable that the proteinase can be classified into the category of prolyl endopeptidase (EC 3.4.21.26), which is the only known enzyme species which can cleave the peptide bond at the carboxy end of a proline residue within the peptide chain [23]. However, SDS-urea-polyacrylamide gel electrophoresis could not detect any fragment of 1.2 kDa or less, which should have been produced by the endopeptidase in the conversion from the 18-kDa protein into the 17-kDa fragment.

Most of the studies on chloroplast proteinases [24–27] are focused on the soluble enzymes probably present in stroma. Only Dalling et al. [28] reported a seemingly thylakoid-bound proteinase in barley chloroplasts. Its activity appeared only in the presence of SDS and was sensitive to SH-group-modifying reagents such as *p*-chloromercuribenzoate and *N*-ethylmaleimide, and a serine proteinase inhibitor, phenylmethylsulphonyl fluoride [28]. Since the proteinase from PS II particles in the present study was insensitive to these inhibitors, it seems to differ from the proteinase reported by Dalling et al.

The N-terminal amino acid sequences and amino acid compositions of the 18-kDa protein and the 17-kDa fragment suggest that the hydrophobic N-terminal region of the 18-kDa protein was lost upon the conversion of 18-kDa protein into 17-kDa fragment. It is remarkable that five proline residues exist in the removed part, and the four of them are in series. It is notable that the values for the molecular mass estimated by SDS-polyacrylamide gel electrophoresis and gel filtration chromatography were similar in the 17-kDa fragment, but distinctly different in the 18-kDa protein. Thus, the hydrophobic N-terminal region which includes a very high proportion of proline residues may distort the steric structure, which increases the effective molecular size if compared with a spherical shape. The removal of this N-terminal region of the 18-kDa protein may convert the protein to a normal one which gives similar values for molecular mass estimated by the different techniques.

The 17-kDa fragment of the 18-kDa protein

lost the functional property of the 18-kDa protein as a Cl^- concentrator. This observation suggests that the N-terminal amino acid sequence of the 18-kDa protein is indispensable for functioning of the protein. The finding that the 17-kDa fragment could not specifically bind to the 24-kDa protein can explain the functional inability of this fragment. It is very likely that the structure of the N-terminal region of the 18-kDa protein given by the hydrophobic amino acid residues and the charged ones at the N-terminal end contributes to this binding as an anchor.

Acknowledgements

The authors are grateful to Ms. Y. Fujimura and Ms. H. Kajiura, National Institute for Basic Biology, for isolation of the 18-kDa protein and its 17-kDa fragment, and also determination of partial amino acid sequences with their skilled techniques. This work was supported by Grants-in-Aid for Cooperative Research (58340037 and 60304093) to T.K. and N.M. from the Ministry of Education, Science and Culture of Japan.

References

1 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539
2 Åkerlund, H.-E. and Jansson, C. (1981) FEBS Lett. 124, 229–232
3 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268
4 Murata, N. Miyao, M., Omata, T., Matsunami, H. and Kuwabara, T. (1984) Biochim. Biophys. Acta 765, 363–369
5 Kuwabara, T. and Murata, N. (1979) Biochim. Biophys. Acta 581, 228–236
6 Kuwabara, T. and Murata, N. (1982) Biochim. Biophys. Acta 680, 210–215
7 Jansson, C., Åkerlund, H.-E. and Andersson, B. (1983) Photosynthesis Res. 4, 271–279
8 Kuwabara, T. and Murata, N. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. 1, pp. 371–374, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht
9 Jansson, C. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. 1, pp. 375–378, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht
10 Akabori, K., Imaoka, A. and Toyoshima, Y. (1984) FEBS Lett. 173, 36–40
11 Miyao, M. and Murata, N. (1985) FEBS Lett. 180, 303–308
12 Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93
13 Kuwabara, T., Miyao, M., Murata, T. and Murata, N. (1985) Biochim. Biophys. Acta 806, 283–289
14 Laemmli, U.K. (1970) Nature 227, 680–685
15 Blombäck, B., Blombäck, M., Edman, P. and Hessel, B. (1966) Biochim. Biophys. Acta 115, 371–396
16 Zimmerman, C.L., Appella, E. and Pisano, J.J. (1977) Anal. Biochem. 77, 569–573
17 Moore, S. (1963) J. Biol. Chem. 238, 235–237
18 Goodwin, T.W. and Morton, R.A. (1946) Biochem. J. 40, 628–632
19 Kuwabara, T. and Murata, N. (1983) Plant Cell Physiol. 24, 741–747
20 Otavsky, W.I. and Drysdale, J.W. (1975) Anal. Biochem. 65, 533–536
21 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
22 Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260
23 Wilk, S. (1983) Life Sci. 33, 2149–2157
24 Ragster, L.E. and Chrispeels, M.J. (1981) Plant Physiol. 67, 104–109
25 Lin, W. and Wittenbach, V.A. (1981) Plant Physiol. 67, 969–972
26 Heck, U., Martinoia, E. and Matile, P. (1981) Planta 151, 198–200
27 Thomas, H. and Huffaker, R.C. (1981) Plant Sci. Lett. 20, 251–262
28 Dalling, M.J., Tang, A.B. and Huffaker, R.C. (1983) Z. Pflanzenphysiol. 111, 311–318