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Biochimica et Biophysica Acta 1320 (1997) 17–26

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Identification of domains on the 43 kDa chlorophyll-carrying protein (CP43) that are shielded from tryptic attack by binding of the extrinsic 33 kDa protein with Photosystem II complex

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Received 7 October 1996; revised 13 January 1997; accepted 16 January 1997

Abstract

The structural association of the spinach 33 kDa extrinsic protein with the 43 kDa chlorophyll-carrying protein (CP43) in oxygen-evolving photosystem II (PS II) complexes was investigated by comparing the peptide mappings and N-terminal sequences of the trypsin-digested products of NaCl-washed PS II membranes, which bind the 33 kDa protein, with those of $CaCl_2$-washed PS II membranes, which lack the 33 kDa protein. (1) Peptide from N-terminus to Arg26 of CP43, which is exposed to stromal side, was digested in both PS II membranes, independent of binding of the 33 kDa protein. (2) Peptide bond of Arg357-Phe358 located in the large extrinsic loop E of CP43, which is exposed to lumenal side, was cleaved by trypsin in $CaCl_2$-washed PS II membranes but not in NaCl-washed PS II membranes. This indicates that the region around Arg357-Phe358 in loop E of CP43 is shielded from tryptic attack by binding of the 33 kDa protein to PS II. (3) Trypsin treatment of $CaCl_2$-washed PS II membranes also cleaved peptide bond between Lys457 and Gly458 in C-terminal region of CP43, while no cleavage of this region was detected by trypsin treatment of NaCl-washed PS II membranes. This implies that a conformational change of the C-terminal region of CP43 which is exposed to stromal side occurred upon removal of the 33 kDa protein, which makes the C-terminal region accessible to trypsin. (4) Release of peptide from Gln60 to C-terminus of the α-subunit of cytochrome *b*-559 was detected only in trypsin treatment of $CaCl_2$-washed PS II membranes, indicating that the C-terminal region of this subunit is shielded from tryptic attack by binding of the 33 kDa protein. (5) The PS II membranes, in which Arg357-Phe358, Lys457-Gly458 of CP43 and the C-terminal part of the cytochrome *b*-559 α-subunit had been cleaved by trypsin, was no longer able to bind the 33 kDa protein. This strongly

Abbreviations: CBB, Coomassie brilliant blue R-250; chl, chlorophyll; CP43, 43 kDa chlorophyll-carrying protein of Photosystem II; CP47, 47 kDa chlorophyll-carrying protein of Photosystem II; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HPLC, high-performance liquid chromatography; LHC, light-harvesting chlorophyll proteins; PS II, Photosystem II; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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PII S0005-2728(97)00005-4

suggests that a domain in loop E of CP43 and/or the C-terminal region of the cytochrome *b*-559 α-subunit are necessary for binding of the extrinsic 33 kDa protein to PS II.

Keywords: CP43; Extrinsic 33 kDa protein; Cytochrome *b*-559; Trypsin; Photosystem II

1. Introduction

The extrinsic 33 kDa protein, referred to as the Mn-stabilizing protein, is present in PSII from cyanobacteria to higher plants and plays an important role in stabilizing binding and maintaining functional conformation of the Mn cluster present at the catalytic center of the H_2O-splitting enzyme (for reviews, see [1],[2]). The association of this protein with PSII intrinsic proteins involves both electrostatic interaction and hydrogen bonding, as it can be released from PSII particles by high concentrations of divalent cations [3] or urea plus NaCl [4]. Removal of the 33 kDa protein from PSII results in a loss of oxygen evolution which can be restored by re-binding of the protein [4–7].

The association of the 33 kDa protein with PSII intrinsic components has been studied in various ways. Most of the evidence so far obtained points to an association of the 33 kDa protein with CP47, one of the chl-binding proteins present in the PSII reaction center core complex. CP47 is an core antenna functioning in transfer of excitation energy to the reaction center [8]. In addition to this role, strong evidence has accumulated suggesting that this protein structurally interacts with the oxygen-evolving site of PS II [9–19]. Based on hydropathy analysis of the derived amino acid sequence of CP47, Bricker proposed that the protein possesses 6 transmembrane helices with three extrinsic loops, loops A, C and E, exposed to lumenal surface and two extrinsic loops, loops B and D, exposed to stromal surface, of the thylakoid membrane [20]. A close association of the 33 kDa protein with CP47 has been demonstrated in crosslinking experiments with various bifunctional reagents [9,10,12,21]. We have reported that all the 33 kDa protein can be covalently and stoichiometrically crosslinked to CP47, without any significant effects on the oxygen-evolving activity, by treatment of oxygen-evolving PS II membranes with a water-soluble carbodiimide, EDC, which is a zero-length crosslinker that catalyses the formation of peptide bond between carboxyl and amino groups in van der Waals contact [14]. Crosslinking of the 33 kDa protein with CP47 prevents release of the protein upon $CaCl_2$ or urea/NaCl wash or heat treatment, thereby stabilizing binding of the Mn cluster and oxygen evolution against these treatments [14–16]. Odom and Bricker [22] analyzed the N-terminal amino acid sequence of peptides obtained from cleavage of EDC-crosslinked products with CNBr and showed that the large extrinsic loop E of CP47 is crosslinked to the N-terminal region of the 33 kDa protein. Recently, we reported that, in addition to crosslinking of the 33 kDa protein with the loop E of CP47, a domain of Lys60-Lys137 on the 33 kDa protein was crosslinked to the extrinsic loop A of CP47, by N-terminal amino acid sequence analysis of protease digests of the EDC-crosslinked products [23]. Interaction of the 33 kDa protein with the loop E of CP47 was also demonstrated by binding assay with a monoclonal antibody [24], protease treatment [24], labeling with N-hydroxysuccinimidobiotin [10] and deletion mutagenesis [17–19].

The 33 kDa protein was also found to crosslink with the α-subunit of cytochrome *b*-559 and the *psb*I gene product [25]. Photoaffinity crosslinking studies suggested that the 33 kDa protein is associated with D1 and D2, the PS II reaction center proteins [26]. These results suggested that the 33 kDa protein is associated with, or is in close proximity to, most of the intrinsic PS II components.

CP43 is another chl-carrying protein functioning as a core antenna in transfer of excitation energy to reaction center. The possible interaction of 33 kDa protein with CP43 has also been implicated from trypsin-digestion experiments. Depletion of the 33 kDa protein from PSII membranes specifically and significantly increased the sensitivity of CP43 towards trypsin digestion, which implies that CP43 is shielded by the 33 kDa protein against proteolytic attack [27]. No information, however, has been reported as to which part of CP43 interacts with the 33 kDa protein.

A topological model similar to that of CP47, has been proposed for CP43 based on hydropathy analysis of derived amino acid sequence [20] and on the pattern of proteolytic digestion detected by peptide-specific antibodies [28]. According to this model, CP43 possesses six transmembrane helices with three extrinsic loops, loops A, C and E, exposed to lumenal surface and two extrinsic loops, loops B and D, exposed to stromal surface. In the present work, we compared the trypsin-digestion pattern of NaCl-washed PSII membranes which were depleted of the 23 and 17 kDa proteins, with that of $CaCl_2$-washed PSII membranes which were depleted of all the three extrinsic proteins of 33, 23 and 17 kDa. The results confirmed that removal of the 33 kDa protein specifically and remarkably accelerates the trypsin digestion of CP43 without affecting the digestion of other PSII intrinsic components significantly. The trypsin-cleavage sites created by the removal of the 33 kDa protein were determined to be Arg357-Phe358 at the large extrinsic loop E exposed to lumenal side, and Lys457-Gly458 near C-terminus which is proposed to be exposed to stromal side, of CP43. In addition, the C-terminal region of α-subunit of cytochrome *b*-559 was cleaved by trypsin upon release of the 33 kDa protein. Reconstitution experiments implied that the loop E of CP43 and/or the C-terminus of cytochrome *b*-559 α-subunit, are necessary for binding of the 33 kDa protein.

2. Materials and methods

Oxygen-evolving PS II membranes were prepared from spinach as in [29], with slight modifications as described in [14]. The membranes were suspended in a medium containing 40 mM MES-NaOH (pH 6.5), 0.4 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl (medium A). PSII membranes depleted of the 23 and 17 kDa extrinsic proteins but retained the 33 kDa protein, were prepared by 1 M NaCl wash as described in [30,31]. PS II membranes depleted of all the extrinsic proteins of 33, 23 and 17 kDa were prepared by 1 M $CaCl_2$ wash as described in [3]. Chlorophyll concentration was determined by the method of Porra et al. [32].

For trypsin treatment, NaCl-washed or $CaCl_2$-washed PS II membranes were suspended in an assay medium containing 40 mM MES-NaOH (pH 6.0), 0.4 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl and incubated with bovine pancreas trypsin (type III, Sigma) for 5 min at 25°C in the dark at a chlorophyll concentration of 1 mg/ml. The reaction was stopped by an addition of 1.6 mM phenylmethylsulfonyl fluoride and subsequently centrifuged at $35\,000 \times g$ for 10 min to separate PS II membranes and peptides released by the trypsin treatment. The resultant pellet (PS II membranes) was resuspended in medium A and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), with a slab gel of 11.5% acrylamide containing 6 M urea in the buffer system of Laemmli [33]. Samples were treated with 5% lithium dodecylsulfate and 75 mM dithiothreitol for 30 min at room temperature prior to electrophoresis. After electrophoresis, gels were stained with CBB and photographed. For immunoblotting and N-terminal amino acid sequencing, proteins in the gels were electrotransferred to a nitrocellulose membrane and a PVDF membrane, respectively. The former was reacted with antibodies raised against CP47, CP43, the 33 kDa protein, D1, D2, apoproteins of LHC II, α-subunit of cytochrome *b*-559 from spinach and visualized with biotinylated anti-rabbit IgG, as described previously [21,25]. Proteins electrotransferred to the PVDF membrane were sequenced automatically with a protein sequencer (Applied Biosystems, model 477A).

Peptides released by trypsin treatments were analyzed by a reversed-phase HPLC column (Waters RP-C4, 3.9×150 mm) equipped on a Shimadzu LC-9A HPLC setup with a built-in STD-10AV UV-VIS detector. Peptides were eluted with a 0–75% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid and monitored at 210 nm.

For reconstitution experiments, the 33 kDa protein was extracted from spinach PS II membranes by 1 M $CaCl_2$ and purified by ion-exchange chromatography with a DEAE-Sepharose CL-6B column, according to Kuwabara et al. [34]. NaCl-washed and $CaCl_2$-washed PS II membranes which had been treated with different concentrations of trypsin were washed with 2.6 M urea plus 0.2 M NaCl to remove the 33 kDa protein [4], and then incubated with the purified 33 kDa protein at a protein to chlorophyll ratio of 0.6 (w/w), in medium A at 0°C in the dark for 30 min. The reconstituted PS II membranes were collected by

centrifugation at $35\,000 \times g$ for 10 min and then washed once with and resuspended in medium A. The amounts of the 33 kDa protein rebound by the reconstitution were determined from the staining intensity of SDS-PAGE of the reconstituted PS II membranes and of the 33 kDa protein released from the reconstituted PS II membranes by 2.6 M urea/0.2 M NaCl treatment.

3. Results

Fig. 1 shows polypeptide patterns of NaCl-washed and $CaCl_2$-washed PS II membranes which were treated with different concentrations of trypsin at 25°C for 5 min. Note that CP43 appears as two closely adjacent split bands in our control PS II (lane 1, Fig. 1); this is also seen in many PS II preparations from higher plants. We tried to determine the identity of these two split bands by N-terminal sequencing but both bands were blocked at their N-termini. This indicates that both of the split bands are CP43, since N-terminus of the native CP43 has been reported to be blocked [35]. Presumably, CP43 is phosphorylated to different extents in our PS II, which then gave rise to the two split bands upon electrophoresis. When NaCl-washed PS II membranes were treated with trypsin, the band intensity of CP43 gradually decreased and a band with an apparent molecular mass of 40 kDa which migrated slightly faster than the original two split bands of CP43 appeared as trypsin concentration was raised. The intensity of LHC II apoprotein bands also decreased slightly and some digestion products appeared at low molecular weight regions. No significant release and digestion of the 33 kDa protein as well as other PS II components were observed upon the trypsin treatment under the conditions employed in Fig. 1. In contrast, when $CaCl_2$-washed PS II membranes were treated with trypsin, the original band of CP43 disappeared much faster and a new band labeled A having an apparent molecular mass of 35 kDa appeared in addition to the 40 kDa trypsin-digestion band, while the polypeptide pattern of other digestion products remained essentially the same as those observed in NaCl-washed PS II membranes. This indicates that removal of the 33 kDa protein specifically and remarkably accelerated the trypsin digestion of CP43, suggesting that binding of the 33 kDa protein shielded CP43 from tryptic attack. This result is in agreement with that reported by Isogai et al. [27] previously. Treatment of the NaCl-washed PS II membranes with trypsin at concentrations higher than 75 μg/ml or for longer times than 10 min caused digestion of the 33 kDa protein itself (data not shown); therefore, for investigation of the shielding effects by binding of the 33 kDa protein on PS II intrinsic proteins against tryptic attack, the treatment was performed at trypsin concentrations below 50 μg/ml for 5 min under which the 33 kDa protein was not affected, in the present study.

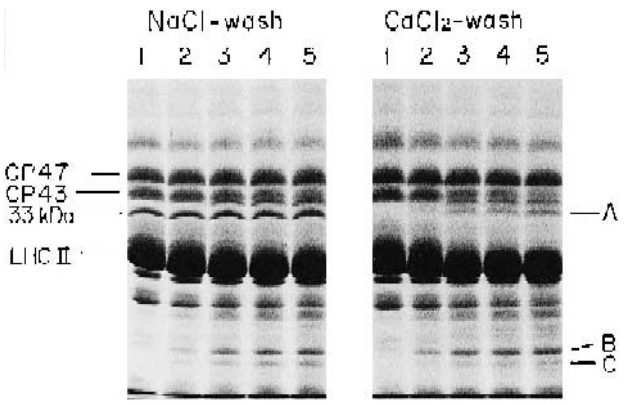

Fig. 1. SDS polyacrylamide gel electrophoresis of trypsin-treated PS II membranes. NaCl-washed and $CaCl_2$-washed PS II membranes were treated with 0 (lane 1), 6.25 (lane 2), 12.5 (lane 3), 25 (lane 4) and 50 (lane 5) μg/ml of trypsin for 5 min at 25°C. The gel was stained with CBB. The bands labeled A, B and C indicate trypsin-digestion products. For other details, see text.

In order to identify the trypsin-digestion products, proteins resolved by the SDS-PAGE were probed by immunoblotting with seven antisera raised against the apoproteins of CP47, CP43 and LHC II, the extrinsic 33 kDa protein, the D2 and D1 proteins, and the 9.4 kDa α-subunit of cytochrome *b*-559. Fig. 2 shows immunoblots with the antiserum against CP43. The antiserum crossreacted with the original band of CP43 (CP43-1), the 40 kDa band (CP43-2) and the 35 kDa band (A band). This indicates that both bands CP43-2 and A are digestion products of CP43. In the bands appeared at low molecular weight regions by trypsin treatment, the B and C bands labeled in Fig. 1 crossreacted with antisera against the apoproteins of LHC II and the D2 protein, respectively, while no other digestion bands were found to crossreact with the seven antisera checked in this work (data not shown).

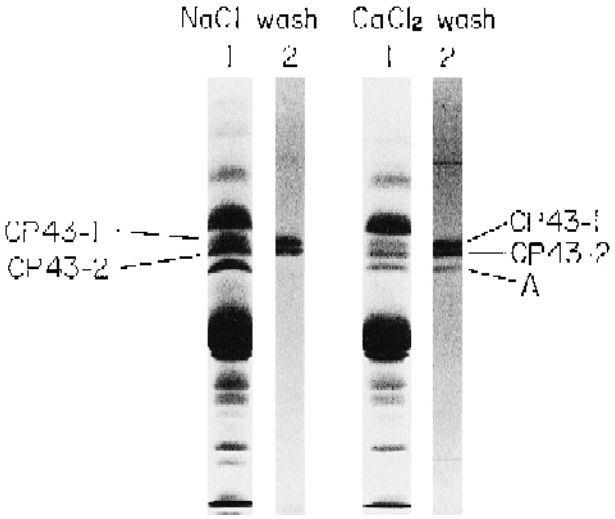

Fig. 2. Identification of trypsin-digested products by immunoblot analysis with antiserum against CP43. NaCl-washed and $CaCl_2$-washed PS II membranes were treated with 50 μg/ml of trypsin for 5 min at 25°C and applied to SDS-PAGE. Lane 1, staining with CBB; lane 2, immunoblotting with antiserum raised against CP43. The bands labeled CP43-2 and A are the trypsin-digested products. Band A corresponds to that labeled in Fig. 1.

In order to determine cleavage sites of CP43 by trypsin treatment, N-terminal amino acid sequences of the CP43-2 and A bands were determined. As shown in Table 1, both CP43-2 bands obtained from NaCl-washed and $CaCl_2$-washed PS II membranes and the A band obtained from $CaCl_2$-washed PS II membranes had the same amino acid sequence; this sequence completely agreed with the sequence from Asp27 to Asn44 of CP43. This indicates that these bands are digestion bands resulted from cleavage of peptide bond between Arg26 and Asp27 of CP43 which is predicted to be exposed to stromal side [20]. The apparent molecular masses of CP43-2 (40 kDa) and A (35 kDa) bands imply that the former is a peptide from Asp27 to C-terminus and the latter is a peptide which was further cleaved probably at the loop E exposed to lumenal side of CP43. For determination of the second cleavage site, C-terminal amino acid sequence of the 35 kDa band (A band) was examined by carboxypeptidase analysis and its C-terminus was found to be Arg but, unfortunately, the second amino acid residue was failed to be determined in this work. Therefore, we analyzed peptides released in solution by trypsin treatment. $CaCl_2$-washed and NaCl-washed PS II membranes were treated with 50 μg/ml of trypsin for 5 min and then centrifuged at 35 000 $\times g$ for 10 min. The polypeptides released into supernatants were separated by reversed-phase HPLC and their resultant peptide maps are shown in Fig. 3. Three peptide peaks (1, 2 and 3 in Fig. 3) were found to be present in $CaCl_2$-washed PS II membranes but not in NaCl-washed PS II membranes. Therefore, these peaks are expected to be the peptides which were shielded by binding of the 33 kDa protein against tryptic attack. By contrast, other peptide peaks which appeared in both NaCl-washed and $CaCl_2$-washed PS II membranes, are considered to be digestion products of the apoproteins of LHC II, the D2 protein, CP43 and probably some other subunits of PS II, which were digested by trypsin irrespective of binding of the 33 kDa protein.

The peptides present in the three peaks, peaks 1, 2 and 3 shown in Fig. 3, were analyzed for their N-terminal amino acid sequences. As shown in Table 1, the N-terminal sequence of peak 1 agreed with amino acid sequence from Gly458 to C-terminus of CP43. This indicates that peptide bond between

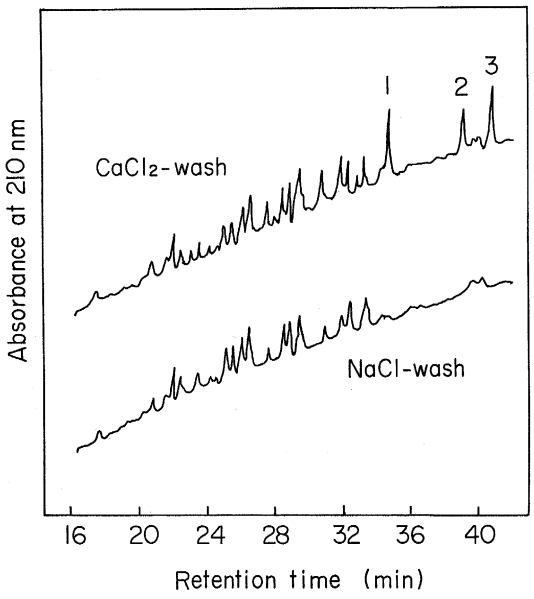

Fig. 3. HPLC elution pattern of the digestion products released into solution by trypsin treatment of PSII membranes. NaCl-washed and $CaCl_2$-washed PS II membranes were treated with 50 μg/ml of trypsin for 5 min at 25°C and then centrifuged. The supernatants were subjected to reversed-phase HPLC and the elution pattern was recorded by monitoring absorbance at 210 nm. For other details, see text.

Table 1
N-terminal amino acid sequences of the trypsin-digested products of bands CP43-2, A labeled in Fig. 2, peaks 1, 2 and 3 shown in Fig. 3, and their assignments

CP43-2	1				5				10				15		
and A bands	D	Q	E	T	T	G	F	A	W	W	A	G	N	A	R	L	I	N
	27				31				36				41		
CP43	D	Q	E	T	T	G	F	A	W	W	A	G	N	A	R	L	I	N
	1				5				10				15		
Peak 1	G	I	D	R	D	F	E	P	V	L	S	M	T	P	L	N		
	458				462				467				472		
CP43	G	I	D	R	D	F	E	P	V	L	S	M	T	P	L	N-COOH		
	1				5				10				15				20	
Peak 2	X	X	X	X	L	I	T	G	R	F	D	S	L	E	Q	L	D	E	F	S	R
	60				64				69				74				79	
b-559 (α)	Q	G	I	P	L	I	T	G	R	F	D	S	L	E	Q	L	D	E	F	S	R
	1				5				10				15				20	
Peak 3	F	W	D	L	R	A	P	W	L	E	P	L	R	G	P	N	G	L	D	L	S	R
	358				362				367				372				377	
Loop E of CP43	F	W	D	L	R	A	P	W	L	E	P	L	R	G	P	N	G	L	D	L	S	R

The measured N-terminal sequences of each digested products shown above were assigned to the corresponding sequences in the known PS-II proteins shown below.

Lys457 and Gly458 of CP43 was cleaved and the C-terminal peptide starting from Gly458 was released into solution only by trypsin treatment of $CaCl_2$-washed PS II membranes. This indicates that removal of the 33 kDa protein makes the C-terminal region of CP43 accessible to trypsin, thus suggesting that protein structure of the C-terminal region of CP43 predicted to be exposed to stromal surface of the thylakoid membrane is affected by binding of the 33 kDa protein at the lumenal side of PS II.

N-terminal sequence analysis of peak 2 showed that this peak is a peptide from Gln60 to Arg80 of α-subunit of cytochrome *b*-559, although amino acid residues from N-terminus to the fourth could not be determined unambiguously due to contamination of residues from unknown peptides (Table 1). This indicates that C-terminal region of α-subunit of cytochrome *b*-559 is shielded from tryptic attack by binding of the 33 kDa protein. This result is consistent with the view that the C-terminus of α-subunit of cytochrome *b*-559 is exposed to the lumenal side and partly shielded by the three extrinsic proteins of 33 kDa, 23 kDa and 17 kDa [36,37].

N-terminal sequence of peak 3 completely agreed with amino acid sequence from Phe358 to Arg379 in the loop E of CP43 (Table 1). This indicates cleavage of the peptide bond between Arg357 and Phe358 by trypsin upon removal of the 33 kDa protein. Since loop E ends at Arg423 and the residues following Arg423 are proposed to be located at the sixth transmembrane helix [28], it is expected that the peptide released into solution due to cleavage of this site by the trypsin treatment is a fragment from Phe358 to certain residue before Arg423 (including the Arg423 itself) in the loop E of CP43 which is further cleaved by trypsin, taking into consideration that the transmembrane helix would remain bound to the membrane and not be released into solution. This second cleavage site may be one of the six potential trypsin-cleavable sites in the sequence from Arg379 to Arg423: Arg379, Lys381, Lys382, Arg390, Arg391 and Arg423 (Fig. 5). Irrespective of where the second cleavage site is, the results imply that the 35 kDa peptide (A band) appeared only in trypsin treatment

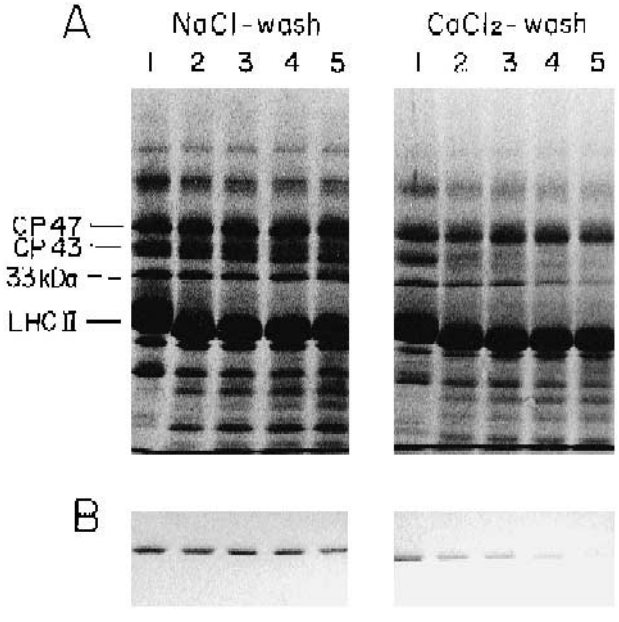

Fig. 4. Reconstitution of the 33 kDa protein with trypsin-treated PS II membranes. NaCl-washed and $CaCl_2$-washed PS II membranes were treated with 0 (lane 1), 6.25 (lane 2), 12.5 (lane 3), 25 (lane 4) and 50 (lane 5) μg/ml of trypsin and then washed with 2.6 M urea and 0.2 M NaCl. The urea/NaCl-washed PS II membranes were reconstituted with the purified 33 kDa protein. A: polypeptide patterns of reconstituted PS II membranes; B: the 33 kDa protein extracted from reconstituted PS II membranes shown in panel A by urea/NaCl wash. For other details, see Section 2.

of $CaCl_2$-washed PS II membranes (see Fig. 1 and Fig. 2) is a peptide from Asp27 to Arg357 of CP43, as the molecular mass of the peptide Asp27-Arg357 is predicted to be 35 948 Da.

The above results showed that, two peptides, one starting from Phe358 of loop E and another from Gly458 to C-terminus located at the stromal side, of CP43, and one peptide starting from Gln60 of the α-subunit of cytochrome *b*-559, were specifically released into solution by the trypsin treatment upon removal of the 33 kDa protein from PS II membranes. In order to examine whether release of these peptides had affected rebinding of the 33 kDa protein to PSII, NaCl-washed and $CaCl_2$-washed PS II membranes were treated with trypsin and then washed with 2.6 M urea and 0.2 M NaCl. The urea/NaCl-washed membranes were then reconstituted with the purified 33 kDa protein. As shown in Fig. 4A, while the NaCl-washed, trypsin-treated PS II membranes were able to rebind the 33 kDa protein efficiently even after treatment with 50 μg/ml trypsin, the rebinding ability of the $CaCl_2$-washed, trypsin-treated PS II membranes decreased significantly as the trypsin concentration was raised. Since the 35 kDa digestion band comigrates with the 33 kDa protein, the amounts of the 33 kDa protein rebound were estimated by extracting the protein from the reconstituted membranes and comparing their staining intensities. Fig. 4B shows the staining intensity of the 33 kDa protein extracted from each reconstituted membrane shown in Fig. 4A. The data clearly confirms that, while the rebinding ability of the NaCl-washed PSII membranes did not change much upon treatment with various concentrations of trypsin, the rebinding capability of the $CaCl_2$-washed PS II membranes decreased significantly by treatment of trypsin at higher concentrations. Upon treatment of trypsin at 50 μg/ml, the $CaCl_2$-washed PSII membranes almost lost its rebinding capability for the 33 kDa protein completely. These results strongly suggest that either one or two or all of the three peptides released by the trypsin treatment in the absence of the 33 kDa protein are required for binding of the 33 kDa protein.

4. Discussion

The present study showed that, among the PSII intrinsic components, CP43 is most remarkably digested by trypsin upon removal of the 33 kDa pro-

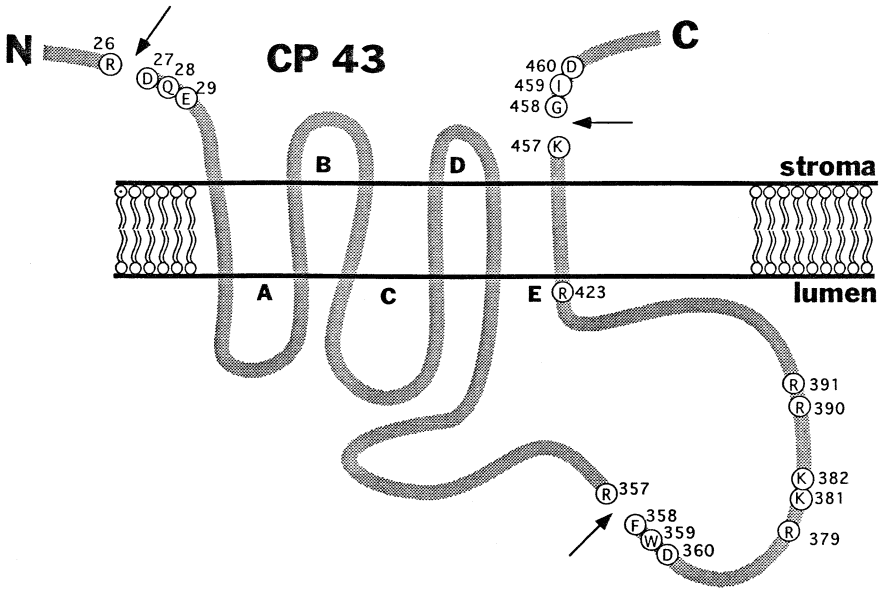

Fig. 5. Schematic diagram of trypsin-cleavage sites on CP43, based on the six transmembrane helices model of CP43 [20,27]. Arrowhead indicates the cleavage sites by trypsin, and the boxed residues in the region Arg379-Arg423 indicate six potential trypsin-cleavable sites. For other details, see text.

tein; the digestion of other PSII components was only marginally either in the presence or absence of the 33 kDa protein. This is in agreement with the previous report that the digestion of CP43 by trypsin is specifically and remarkably accelerated by removal of the 33 kDa protein [27]. Thus, binding of the 33 kDa protein to PSII shielded CP43 from accessing of trypsin, which implies that the 33 kDa protein is located near CP43. This is the only evidence so far reported suggesting a close relationship of the 33 kDa protein with CP43.

The site cleaved off by trypsin on CP43 was determined to be Arg26-Asp27 either in the presence or absence of the 33 kDa protein. Upon removal of the 33 kDa protein, two additional sites on CP43 were cleaved off by trypsin; these two sites were determined to be Arg357-Phe358 and Lys457-Gly458. Fig. 5 depicts a schematic diagram of CP43 showing these cleavage sites. According to the six transmembrane helices model [20], residues Arg357-Phe358 are located in the large extrinsic loop E exposed to lumenal side, whereas residues Lys457-Gly458 are located in the C-terminal part of CP43 which is exposed to the stromal side. Since the 33 kDa protein is associated with the lumenal side of PSII, the results suggest that a region around Arg357-Phe358 at loop E of CP43 is closely related to the 33 kDa protein. Consequently, the site around Lys457-Gly458 does not likely interact with the 33 kDa protein directly; instead, it is possible that removal of the 33 kDa protein at the lumenal side induced a conformational change of the CP43 at the stromal side which then makes the site of Lys457-Gly458 liable to cleavage by trypsin. In fact, an effect on properties of the acceptor side of PSII upon either biochemical removal of the 33 kDa protein [38] or genetic deletion of the *psb*O gene encoding the 33 kDa protein [39,40] has been previously reported based on thermoluminescence and fluorescence measurements. Thus, it is possible that this effect on the acceptor side of PSII is caused, at leased partly, by a conformational change of CP43 upon removal of the 33 kDa protein.

In addition to CP43, removal of the 33 kDa protein caused cleavage of the peptide bond between Arg59 and Gln60 of the α-subunit of cytochrome *b*-559 by trypsin, resulting in the release of a peptide from Gln60 to C-terminus of the cytochrome *b*-559 α-subunit. This confirms the previous findings of Tae et al. [36,37] that the C-terminus of this subunit is exposed to the lumenal side of thylakoids and removal of the three extrinsic proteins results in an increase of the accessibility of the subunit to trypsin. Furthermore, we have reported previously that the cytochrome *b*-559 α-subunit is crosslinked with the 33 kDa protein [25]. It is thus possible that the crosslinking site is located in the region around Arg59-Gln60 of the C-terminal part of the cytochrome subunit.

Cleavage of the peptide bond at Arg26-Asp27 of CP43 resulted in a product with an apparent molecular mass of 40 kDa, irrespective of the binding of the 33 kDa protein. Accordingly, a peptide from N-terminus to Arg26 of CP43 must be released into solution by the trypsin treatment, as this part is predicted to be exposed to the stromal side, not spanning the membrane of the lipid bilayer (Fig. 5). The fact that no release of the 33 kDa protein from NaCl-washed PSII membranes was detected upon treatment with trypsin indicates that the association of the 33 kDa protein with PSII was not affected by removal of the N-terminal part of CP43. This is consistent with the result that the NaCl-washed, trypsin-treated PSII in which, CP43 was cleaved off only at the site of Arg26-Asp27, was still able to rebind the 33 kDa protein completely. In contrast, trypsin digestion of $CaCl_2$-washed PSII membranes led to a loss of the rebinding ability of the PSII for the 33 kDa protein. As discussed above, the C-terminal part of CP43 that is released into solution by cleavage at the site of Lys457-Gly458 by trypsin in the absence of the 33 kDa protein, is located in the stromal side and thus not likely to interact with the 33 kDa protein directly. Consequently, the results imply that the peptide starting from Phe358 at the large extrinsic loop E of CP43 which is released into solution by trypsin cleavage at Arg357-Phe358 in the absence of the 33 kDa protein, and/or the C-terminal part of cytochrome *b*-559 α-subunit released into solution by trypsin cleavage at Arg59-Gln60 in the absence of the 33 kDa protein, are required for binding of the 33 kDa protein. Although we could not determine the C-terminus of the peptide starting from Phe358 of CP43 released into solution by trypsin, it is most probably that it terminates at one of the six potential trypsin-cleavable sites before Arg423 (including Arg423) (Fig. 5) which is further cleaved by

trypsin, as the residues immediately beyond Arg423 are proposed to be the start point of the sixth transmembrane helix [28] which would not be released into solution even if it is cleaved by trypsin. Thus, the results suggest that a domain around Arg357-Phe358 in the large extrinsic loop E of CP43 is required for binding of the 33 kDa protein. In relation to this, it is interesting to note that CP47, which has a limited structural homology with CP43, has been reported to interact with the 33 kDa protein also at its large extrinsic loop E.

It is to be emphasized that, in contrast to CP47 which has been shown to interact with the 33 kDa protein from various lines of evidence including crosslinking [9,10,12,21], antibody binding [24] and site-directed mutagenesis [17–19], the interaction of CP43 with the 33 kDa protein has not been shown previously, in particularly in various crosslinking studies. Moreover, Rögner et al. [41] recently proposed a structural model of PSII based on their electron microscopic studies in which, no intimate contact between CP43 and the 33 kDa protein seems to occur. One may argue, therefore, that the increased accessibility of CP43 to trypsin upon removal of the 33 kDa protein may result from a conformational change induced by $CaCl_2$ wash employed to remove the 33 kDa protein and hence does not necessarily imply that CP43 is related with the 33 kDa protein. While we cannot exclude this possibility at present, it should be pointed out that no significant conformational change at the lumenal side of PSII has been reported to occur upon $CaCl_2$ wash. Our present results that while trypsin treatment of NaCl-washed PSII did not affect the rebinding ability of PSII for the 33 kDa protein, the digestion of CP43 by trypsin in the absence of the 33 kDa protein resulted in a significant loss of the rebinding ability of PSII for the 33 kDa protein strongly suggest that CP43 interacts directly with the 33 kDa protein. Moreover, a lack of crosslinking between CP43 and the 33 kDa protein does not necessarily mean that these two subunits are not in close contact, since a successful crosslinking between two subunits requires a close location of appropriate residues crosslinkable from the two subunits, and the residues of CP43 and the 33 kDa protein in contact may not be suitable for crosslinking. Finally, a detailed comparison between our present results and those from electron microscopy studies cannot be made until a proper increase in the resolution of the structural model produced in the latter studies is achieved.

Acknowledgements

We would like to thank Drs. T. Horio and T. Kakuno of Osaka University for providing the antibodies against spinach CP47, CP43, 33 kDa protein and apoproteins of LHC II, Dr. M. Ikeuchi of University of Tokyo for providing the antibodies against spinach D1, D2 and the α-subunit of cytochrome *b*-559. This work was supported in part by a grant-in-aid for Co-operative Research (No. 05304006) from the Ministry of Education, Science and Culture of Japan (I.E.).

References

[1] Ghanotakis, D.F. and Yocum, C.F. (1985) Photosynth. Res. 7, 97–114.
[2] Debus, R.J. (1992) Biochim. Biophys. Acta 1102, 269–352.
[3] Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260.
[4] Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354.
[5] Ono, T. and Inoue, Y. (1984) FEBS Lett. 166, 381–384.
[6] Kuwabara, T., Miyao, M., Murata, T. and Murata, N. (1985) Biochim. Biophys. Acta 806, 283–289.
[7] Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286.
[8] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[9] Enami, I., Satoh, K. and Katoh, S. (1987) FEBS Lett. 226, 161–165.
[10] Bricker, T.M., Odom, W.R. and Queirolo, C.B. (1988) FEBS Lett. 231, 111–117.
[11] Vermaas, W.F.J., Ikeuchi, M. and Inoue, Y. (1988) Photosynth. Res. 17, 97–113.
[12] Enami, I., Miyaoka, T., Mochizuki, Y., Shen, J.-R., Satoh, K. and Katoh, S. (1989) Biochim. Biophys. Acta 973, 35–40.
[13] Rögner, M., Chrisholm, D.A. and Diner, B. (1991) Biochemistry 30, 5387–5395.
[14] Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sonoike, K., Inoue, Y. and Katoh, S. (1991) Biochim. Biophys. Acta 1060, 224–232.
[15] Enami, I., Tomo, T., Kitamura, M. and Katoh, S. (1994) Biochim. Biophys. Acta 1185, 75–80.
[16] Enami, I., Kitamura, M., Tomo, T., Isokawa, Y., Ohta, H. and Katoh, S. (1994) Biochim. Biophys. Acta 1186, 52–58.
[17] Haag, E., Eaton-Rye, J.J., Renger, G. and Vermaas, W.F.J. (1993) Biochemistry 32, 4444–4454.

[18] Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Inoue, Y., Vermaas, W.F.J. and Renger, G. (1994) Biochemistry 33, 12063–12071.
[19] Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Seeliger, A.G., Inoue, Y., Vermaas, W.F.J. and Renger, G. (1995) Biochemistry 34, 6847–6856.
[20] Bricker, T.M. (1990) Photosynth. Res. 24, 1–13.
[21] Enami, I., Mochizuki, Y., Takahashi, S., Kakuno, T., Horio, T., Satoh, K. and Katoh, S. (1990) Plant Cell Physiol. 31, 725–728.
[22] Odom, W.R. and Bricker, T.M. (1992) Biochemistry 31, 5616–5620.
[23] Ohta, H., Yoshida, N., Sano, M., Hirano, M., Nakazato, K. and Enami, I. (1995) in Photosynthesis: From Light to Biosphere (Mathis, P. ed.), Vol. 2, pp. 361–364.
[24] Bricker, T.M. and Frankel, L.K. (1987) Arch. Biochem. Biophys. 256, 295–301.
[25] Enami, I., Ohta, S., Mitsuhashi, S., Takahashi, S., Ikeuchi, M. and Katoh, S. (1992) Plant Cell Physiol. 33, 291–297.
[26] Mei, R., Green, J.P., Sayre, R.T. and Frasch, W.D. (1989) Biochemistry 28, 5560–5567.
[27] Isogai, Y., Yamamoto, Y. and Nishimura, M. (1985) FEBS Lett. 187, 240–244.
[28] Sayre, R.T. and Wrobel-Boerner, E.A. (1994) Photosynth. Res. 40, 11–19.
[29] Berthold, D.A., Babcock, G.T. and Yocum C.F. (1981) FEBS Lett. 134, 231–234.
[30] Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93.
[31] Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173.
[32] Porra, R.J., Thompson, W.A. and Kriedemann, P.E. (1989) Biochim. Biophys. Acta 975, 384–394.
[33] Laemmli, U.K. (1970) Nature 227, 680–685.
[34] Kuwabara, T., Murata, T., Miyao, M. and Murata, N. (1986) Biochim. Biophys. Acta 850, 146–155.
[35] Michel, H., Hunt, D.F., Shabanowitz, J. and Bennett, J. (1988) J. Biol. Chem. 263, 1123–1130.
[36] Tae, G.-S., Black, M.T., Cramer, W.A., Vallon, O. and Bogorad, L. (1988) Biochemistry 27, 9075–9080.
[37] Tae, G.S. and Cramer, W.A. (1994) Biochemistry 33, 10060–10068.
[38] Vass, I., Ono, T. and Inoue, Y. (1987) Biochim. Biophys. Acta 892, 224–235.
[39] Vass, I., Cook, K.M., Deak, Z., Mayes, S.M. and Barber, J. (1992) Biochim. Biophys. Acta 1102, 195–201.
[40] Burnap, R.L., Qian, M., Shen, J.-R., Inoue, Y. and Sherman, L.A. (1994) Biochemistry 33, 13712–13718.
[41] Rögner, M., Boekema, E.J. and Barber, J. (1996) Trends Biochem. Sci. 21, 44–49.