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Two regions of the Mn-stabilizing protein from *Synechococcus elongatus* that are involved in binding to photosystem II complexes

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Abstract

Limited proteolysis of the Mn-stabilizing protein (MSP) from the thermophilic cyanobacterium *Synechococcus elongatus* with chymotrypsin, trypsin or lysylendopeptidase that yielded four major polypeptides of 26 kDa, 22 kDa, 15 kDa and 11 kDa on denaturing gel electrophoresis resulted in total loss of the binding capacity of the protein to PSII complexes. Analyses of electrophoretic patterns and amino-terminal sequences of the proteolytic products revealed that the three proteases specifically cleaved the protein at a site between Phe^{156} and Gly^{163} or between Arg^{184} and Ser^{191}. Site-directed mutagenesis was used to construct two mutant MSPs that had a nick between Phe^{156} and Leu^{157}, a chymotrypsin-cleavage site, and Met before Leu^{157} or in place of Leu^{157}. The two mutant proteins failed to bind to PSII complexes, although they largely retained ordered secondary structure and comigrated with the wild-type proteins in non-denaturing gel electrophoresis. The loss of the protein binding can be ascribed to introduction of a nick because a mutant protein that had Met in place of Leu^{157} but no nick was able to specifically bind to the functional site of PSII complexes and restore the oxygen-evolving activity as effectively as the wild-type protein. In contrast, a mutant MSP with Met inserted between Phe^{156} and Leu^{157} bound only weakly and non-specifically to PSII complexes and failed to reactivate oxygen evolution. Thus, the binding of the protein to the functional site of the PSII complex was highly sensitive to a small structural change that was caused by cleavage or insertion of a single amino acid residue between Phe^{156} and Leu^{157}. The results suggest that the Phe^{156}-Gly^{163} and Arg^{184}-Ser^{191} sequences of the cyanobacterial MSP are regions for interaction with PSII complexes.

Keywords: Binding site to PSII complex; Chymotrypsin; Mn-stabilizing protein; Limited proteolysis; Mutagenesis; (*Synechococcus elongatus*)

1. Introduction

The PSII reaction center complex that catalyzes transport of electrons from H_2O to the plastoquinone pool consists of intrinsic proteins that are called CP47, CP43, the D1 and D2 proteins, the large and small subunits of cytochrome *b*559, and the 4 kDa *psb*I gene product, together with several small polypeptides of unknown functions [1,2]. The PSII complex is also associated with the three extrinsic proteins with apparent molecular masses of 33, 23 and 17 kDa that function in oxygen evolution [3,4]. The

Abbreviations: MSP, extrinsic 33 kDa Mn-stabilizing protein; PSII, photosystem II; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; CBB, Coomassie brilliant blue; MES, 2-(*N*-morpholino)ethanesulfonic acid monohydrate; CD, circular dichronism

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23 and 17 kDa proteins that occur in higher plants and algae but not in cyanobacteria have regulatory roles related to Ca^{2+} and Cl^-. The 33 kDa protein that is found in all oxygenic photosynthetic organisms is required for the optimal operation of the water oxidation reaction. Removal of the 33 kDa protein by treatment with high concentrations of $CaCl_2$ [5] or urea/NaCl [6] results in loss of the oxygen-evolving activity and the lost activity is, at least partly, restored by rebinding of the protein [7,8]. The 33 kDa protein is called Mn-stabilizing protein (MSP) because solubilization of the protein gave rise to gradual release of two out of four Mn atoms that are associated with the water oxidizing center [7].

Crosslinking studies have shown that MSP is located in close proximity of several intrinsic proteins of the PSII complex [9–14] and, in particular, experiments with a zero-length crosslinker have demonstrated that the protein binds directly to CP47 [10–12]. Various attempts have been made to elucidate the molecular link between the MSP and PSII reaction center complex. Removal of 16 amino acid residues from the N-terminal sequence of the spinach MSP by protease digestion resulted in loss of the binding capacity of the protein [15]. More recently, Glu^1-Lys^4 of the spinach MSP was shown to be inaccessible to the bulk solvent when the protein was associated with the PSII complex [16]. These results suggest that the N-terminal sequence is a region of interaction with the PSII complex. Asp^9, the most highly conserved charged residue in the N-terminal sequence could, however, be replaced by Asn or Lys with no significant effect on PSII activities [17–19].

There is evidence that suggests involvement of another domain of the MSP molecule in binding to PSII complexes. Substitution of strictly conserved Asp^{157} of the spinach MSP by Lys resulted in decrease in the capacity of the protein to restore the oxygen-evolving activity of MSP-depleted PSII complexes [20]. Replacement by Asn of Asp^{159} of *Synechocystis* MSP, which corresponds to Asp^{157} in the spinach protein, also caused significant decrease in the rate and stability of oxygen evolution in the cyanobacterial cells [18]. The Lys^{159}-Lys^{186} sequence of the spinach MSP was reported to become inaccessible to an amino group specific reagent on association of the protein with PSII membranes [16]. Miura et al. showed, however, that lysyl residues that were accessible to two modifiers of amino group only when the spinach protein was free in solution distribute over the entire protein molecule [21].

In the present study, we first investigated effects of limited proteolysis of the MSP from the thermophilic cyanobacterium *Synechococcus elongatus* with chymotrypsin, trypsin or lysylendopeptidase on binding of the protein to PSII complexes. Because cleavage of the protein at a single site caused the total loss of its binding capacity, several MSP molecules that have a mutation at a chymotrypsin-cleavage site were constructed and their capability to bind to or reactivate oxygen evolution of MSP-depleted PSII complexes was examined. The results suggest that the two eight-amino acid sequences from Phe^{156} to Gly^{163} and from Arg^{184} to Ser^{191} are regions for interaction with PSII complexes.

2. Materials and methods

The *S. elongatus* MSP was expressed in *Escherichia coli* cells and extracted and purified as in [22]. For limited proteolysis, the protein (2 mg/ml) was incubated with chymotrypsin at a protein/enzyme weight ratio of 1000 in 50 mM Tris/HCl (pH 9.0) and 150 mM NaCl for indicated periods of time at 30°C. The protein was also treated with trypsin [2500:1 (w/w)] in 0.2 M K-phosphate (pH 7.0) or with lysylendopeptidase [10 000:1 (w/w)] in 50 mM Tris/HCl (pH 9.0). The reactions were terminated by addition of 2.5 mM phenylmethylsulfonyl fluoride. Chymotrypsin (45 U/mg), trypsin (209 U/mg) and lysylendopeptidase (10 AU) were the products of Cooper Biochemical (Missouri, USA), Worthington Diagnostic Systems Inc. (Washington, USA) and Wako Pure Biochemical (Tokyo, Japan), respectively.

Site-directed mutagenesis was carried out by the oligonucleotide mutagenesis method. The pET8c expression vector that contains the wild-type *psbO* gene of *S. elongatus* was constructed as in [22]. The *Xba*I/*Bam*HI fragment of the vector that carries the whole *psbO* gene was cloned into the multiple cloning site of pBluescriptII phagemid vector (Stratagene) and subcloned into *Sac*I/*Hin*dIII site of the M13mp18 phagemid vector. Desired mutagenesis was introduced into an M13mp18 template using the Sculp-

Table 1
Design of oligonucleotides used for the site-directed mutagenesis of MSP

Mutants	Mutagenetic oligonucleotides[a,b]
156/end	5′CGTCCTTTGGGATCCTAGAAGTTGGCGGTGC3′
156/M+157	5′TCCTTTGGGATCGAGCATGGTATATCTCCTA-GAAGTTGGCGGTGC3′
156/L157M	5′CCGTCCTTTGGGGTCCATGGTATATCTCCTA-GAAGTTGGCGGTGC3′
L157M	5′CCGTCCTTTGGGGTCCATGAAGTTGGCGGTGCG3′
156+M+157	5′GTCCTTTGGGATCGAGCAT-GAAGTTGGCGGTGCG3′
189/end	5′CCCTTCGTGAGGGATCCTAGCGTTTGA-CATTGGC3′
189/M+190	5′CTTCGTGAGGGAGAACATGGTATATCTCC-TAGCGTTTGACATTGGC3′

[a]The nucleotide sequences are of the complementary strand.

ture in vitro mutagenesis system (Amersham). The mutagenetic oligonucleotides used were shown in Table 1. The single strand DNA of the mutated *psbO* gene was isolated and sequenced to check the mutation. The mutated *psbO* gene was cloned into pET8c expression vector and expressed in *E. coli* cells. The mutant MSPs were purified by the method that was employed for isolation of the wild-type MSP [22].

Oxygen-evolving PSII complexes were isolated from *S. elongatus* [23]. The PSII complexes were treated with 1 M $CaCl_2$ or with 2.6 M urea/0.2 M NaCl for 30 min at 0°C and MSP-depleted PSII complexes were collected by centrifugation at $370\,000 \times g$ for 40 min and suspended in 20 mM MES/NaOH (pH 6.0), 50 mM $CaCl_2$ and 10% glycerol. Binding of MSP to PSII complexes was examined by adding the protein to the washed PSII complexes at indicated ratios of the protein to PSII. A stoichiometry of 45 chlorophyll per PSII was assumed [23]. After incubation at 0°C for 1 h, PSII complexes were recovered by centrifugation at $370\,000 \times g$ for 40 min, once washed with and suspended in 50 mM MES/NaOH (pH 6.0), 10 mM $MgCl_2$ and 25% glycerol and subjected to SDS polyacrylamide gel electrophoresis. Samples were denatured with 5% SDS and 60 mM dithiothreitol and electrophoresis was performed according to the procedure described by Laemmli [24]. After electrophoresis, gels were stained with CBB R-250 for polypeptides and scanned at 560 nm with an Atto densitometer (AE-6900). Abundance of MSP bound to PSII complexes was determined by measuring peak area of MSP bands relative to that of CP47 protein band. Non-denaturing polyacrylamide gel electrophoresis was performed with a 18% polyacrylamide gel that contained no SDS.

The N-terminal amino acid sequence was analyzed with an Applied Biosystems 477A protein sequencer. Polypeptides resolved by SDS gel electrophoresis were semi-dry-blotted at a constant current of 2.0 mA/cm^2 for 1 h onto polyvinylidene difluoride membranes (ProBlott; Applied Biosystems Japan Ltd., Tokyo).

CD spectra were determined with a JASCO J-700A spectropolarimeter. The concentration of sample proteins was 150 μg/ml in 15 mM K-phosphate (pH 6.5).

Oxygen evolution was determined at 40°C with a Clark-type oxygen electrode as in [23]. The reaction medium contained 50 mM MES/NaOH (pH 6.0), 10 mM NaCl, 5 mM $MgCl_2$ and 1 M sucrose. The activity was determined with 0.4 mM 2,6-dichloro-*p*-benzoquinone as electron acceptor.

3. Results

The *Synechococcus* MSP was treated with chymotrypsin, trypsin or lysylendopeptidase for different periods of time and polypeptide fragments produced were analyzed by SDS gel electrophoresis. Irrespective of the enzyme used, four polypeptide fragments of about 26 kDa, 22 kDa, 15 kDa and 11 kDa appeared at early stages of the enzyme-treatment (Fig. 1). The four fragments that were produced by treatment with chymotrypsin for 1 h were referred to C1, C2, C3 and C4 in the order of decreasing molecular masses. The whole protein and C1, together with several weak bands that migrated between them, decreased with increasing time of incubation and loss of the protein and fragments was accompanied by intensification of the C2 band. This suggests that C2 was produced by cleavage of not only the whole protein but also C1 and other large polypeptide fragments. The band intensity of C2 remained thereafter at a level close to the original band intensity of the whole protein. Only a faint band appeared below C2 after 2 h of incubation. The results suggest that C2 was strongly resistant to chymotrypsin so that the

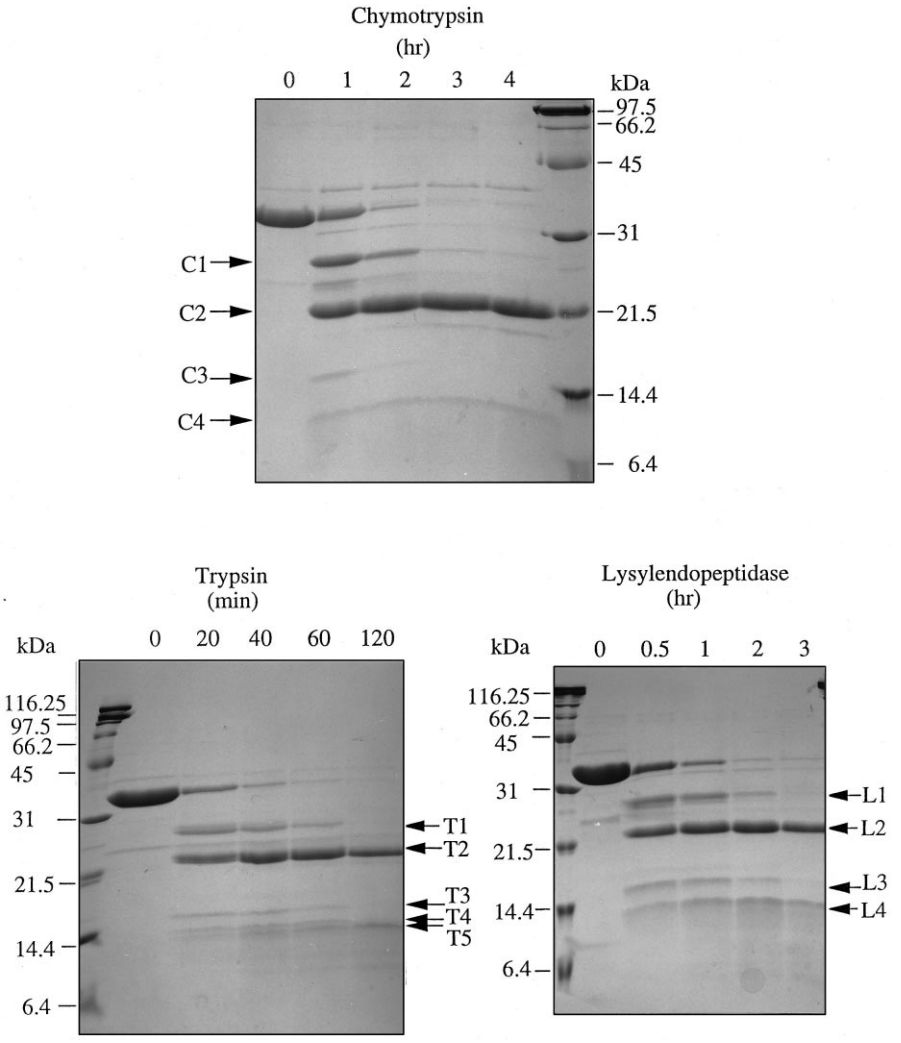

Fig. 1. Limited proteolysis of MSP. The protein was treated with chymotrypsin, trypsin and lysylendopeptidase for indicated periods of time, then polypeptide compositions were analyzed by SDS gel electrophoresis on 20% polyacrylamide gel containing 0.1% SDS. Cx, Tx and Lx are polypeptide fragments produced by chymotrypsin, trypsin and lysylendopeptidase, respectively (see text).

whole protein was quantitatively converted to C2 during incubation with the enzyme. C3 temporarily appeared after 1 h of incubation, then disappeared, whereas C4 attained a maximum level after 2 h of treatment and, thereafter, remained unaltered. The *Synechococcus* MSP that has the molecular mass of 27 kDa [25] migrated at a gel position corresponding to about 35 kDa. The data suggest, therefore, that chymotrypsin first cut the protein at two sites, producing two pairs of fragments, C1 (26 kDa)/C4 (11 kDa) and C2 (22 kDa)/C3 (15 kDa), then shortened C1 and C3 to yield C2 and C4, respectively.

Similar patterns of proteolysis of the cyanobacterial MSP were observed with trypsin and lysylendopeptidase, except that the fastest migrating band produced by trypsin-treatment was split into two bands (Fig. 1). The five bands that were produced by treatment with trypsin for 20 min are called T1, T2, T3, T4 and T5 in the order of increasing mobility. The four fragments that appeared after 30 min of incubation with lysylendopeptidase are L1, L2, L3 and L4 in the same order. Both T1 and L1 gradually decreased with increasing time of incubation and losses of the two largest fragments (and the whole protein) were accompanied by intensification of the T2 and L2 bands. T5 and L4 also increased at the cost of T3 (and T4) and L3, respectively. These results suggest that trypsin and lysylendopeptidase also

Table 2
N-terminal sequences of polypeptide fragments produced by proteolysis with chymotrypsin, trypsin and lysylendopeptidase

Proteases	Fragments	Amino-terminal sequences
Chymotrypsin	C1	AKQTL
	C2	AKQTL
	C3	LDPK
	C4	SLTKG
Trypsin	T1	AKQTLTY
	T2	AKQTL
	T3	GLASGYDSA
	T4	ANVXXFSLT
	T5	FSLTXGQI
Lysylendopeptidase	L1	AKQTLXYDDI
	L2	AKQTLTYDDI
	L3	GXGLASGYD
	L4	XFSLT

X, an unidentified amino acid.

primarily attack the protein at two sites. The band intensities of T2 and L2 were, however, considerably weaker than the original band intensity of the whole protein. This implies that a substantial part of the protein was digested by the two enzymes without yielding T2 and L2.

N-terminal amino acid sequences of the polypeptide fragments produced by the three proteases were determined (Table 2) and the cleavage sites deduced are shown in Fig. 2. The N-terminal sequences of the largest (C1, T1, L1) and the second largest fragments (C2, T2, L2) were identical to that of the whole protein [25]. The N-terminal sequences of C3 and C4 show that chymotrypsin cleaved the protein between Phe^{156} and Leu^{157} or between Phe^{190} and Ser^{191}, thereby producing the C2 (Ala^{1}-Phe^{156})/C3 (Leu^{157}-Ala^{246}) pair or the C1 (Ala^{1}-Phe^{190})/C4 (Ser^{191}-Ala^{246}) pair, respectively. Then, the enzyme cut C1 after Phe^{156} to yield C2, and C3 after Phe^{190} to give C4. The N-terminal sequences of T3, T4 and T5 show that trypsin cut the protein at the C-terminal side of Arg^{162}, Arg^{184} and Arg^{189}. Thus, T1, T2, T3 and T4 correspond to Ala^{1}-Arg^{184}, Ala^{1}-Arg^{162}, Gly^{163}-Ala^{246} and Ala^{185}-Ala^{246}, respectively. Removal of a short peptide (Ala^{185}-Arg^{189}) from T4 yielded T5 (Phe^{190}-Ala^{246}). Lysylendopeptidase attacked the protein at the C-terminal side of Lys^{160} and Lys^{188}. Thus, the L1 (Ala^{1}-Lys^{188})/L4 (Arg^{189}-Ala^{246}) pair and the L2 (Ala^{1}-Lys^{160})/L3 (Gly^{161}-Ala^{246}) pair were the first proteolytic products. Then, the enzyme cleaved between Lys^{160} and Gly^{161} to produce L2 and L4 from L1 and L3, respectively.

We examined whether the protein that was cleaved by the three enzymes is able to bind to PSII complexes isolated from the cyanobacterium. MSP was completely solubilized by treatment of the oxygen-evolving PSII complexes with 1 M $CaCl_2$ (Fig. 3, lane 3). $CaCl_2$-wash also removed allophycocyanin, the linker proteins and the anchor protein and its proteolytic fragments that were associated with the complexes [26]. Washed PSII complexes were fully reconstituted with the untreated MSP (lane 4). When the protein that had been cleaved by chymotrypsin as shown in lane 6 was added, washed complexes bound only the whole protein but none of the proteolytic products (lane 5). The protein that had been cut by trypsin (lane 7) or lysylendopeptidase

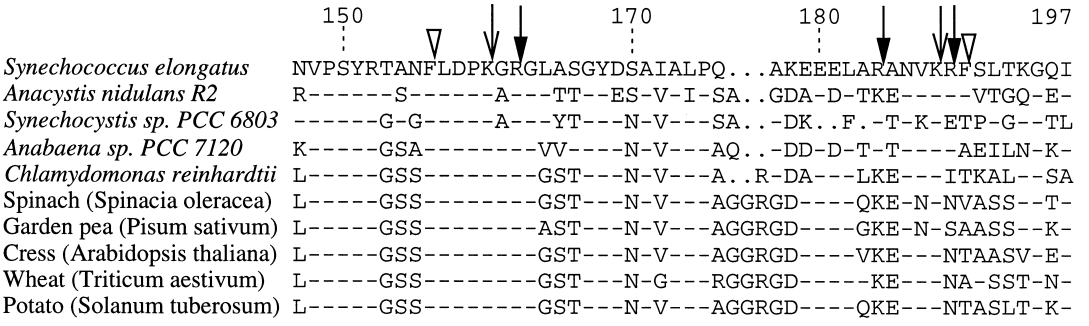

Fig. 2. Proposed cleavage sites of MSP. Triangle, chymotrypsin; bar with triangle, trypsin; arrow, lysylendopeptidase. The corresponding amino acid sequences of MSPs from other oxygenic photosynthetic organisms are also shown. Bars, amino acids the same as the *S. elongatus* protein; dots, amino acids deleted.

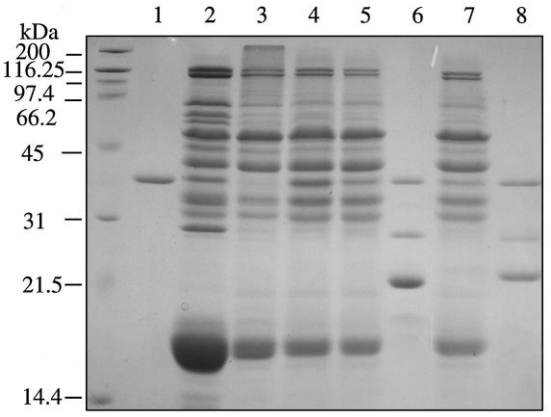

Fig. 3. Effects of limited proteolysis on binding of MSP to PSII complexes. MSP, either untreated or treated with a protease, was added to $CaCl_2$-washed PSII complexes at a protein/PSII ration of 2. After incubation for 1 h at 0°C, the complexes were collected by centrifugation, once washed, then applied to 14% polyacrylamide gels containing 0.1% SDS and 6 M urea after denaturation. Lane 1, MSP; lane 2, PSII complexes; lane 3, $CaCl_2$-washed PSII complexes; lane 4, $CaCl_2$-washed PSII complexes incubated with MSP; lane 5, $CaCl_2$-washed complexes incubated with chymotrypsin-digested MSP; lane 6, chymotrypsin-digested MSP; lane 7, $CaCl_2$-washed complexes incubated with trypsin-digested MSP; lane 8, trypsin-digested MSP. MSP was treated with chymotrypsin and trypsin for 1 h and 20 min, respectively.

(not shown) also failed to bind to PSII complexes. The result suggests that introduction of a nick between Phe^{156}-Gly^{163} or between Arg^{184}-Ser^{191} results in total loss of the binding capacity of the protein to the PSII complex.

When the protein that had been treated with chymotrypsin for 30 min was subjected to non-denaturing gel electrophoresis, two bands were resolved as shown in the upper panels of Fig. 4A. Longer treatment with the enzyme increased the fast-moving band at the cost of the slow-moving band (Fig. 4B). Polypeptide compositions of the two bands were analyzed by second dimensional electrophoresis after denaturation (lower panels). The whole protein, C1, C2, C3 and C4 were resolved from the slow-moving band. As will be shown below, C2 and an N-terminal fragment that corresponds to C1 from which Phe^{190} was deleted migrated differently from the whole protein in non-denaturing gels (see Fig. 5). Comigration of the four fragments with the whole protein, therefore, indicates that C1 and C2 were still associated with C4 and C3, respectively. The fast-moving band yielded only C2 and C4 on the second dimensional gel electrophoresis. This suggests that a polypeptide segment, Leu^{157}-Phe^{190}, is not essential for the mutual association of C2 and C4. Thus, the loss of the binding capacity that was caused by limited proteolysis cannot be ascribed to dissociation of the resulting polypeptide fragments.

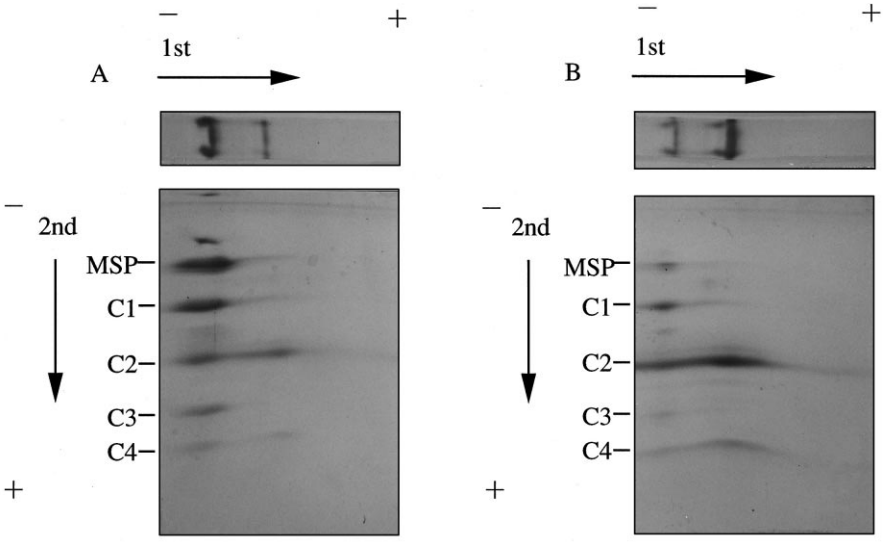

Fig. 4. Two-dimensional polyacrylamide gel electrophoresis of MSP treated with chymotrypsin. Upper panels, non-denaturing gel electrophoresis of MSP that was treated with chymotrypsin for 30 min (A) and 2 h (B). Lower panels, second dimensional SDS gel electrophoresis of the two bands resolved in the first one-dimensional electrophoresis after denaturation.

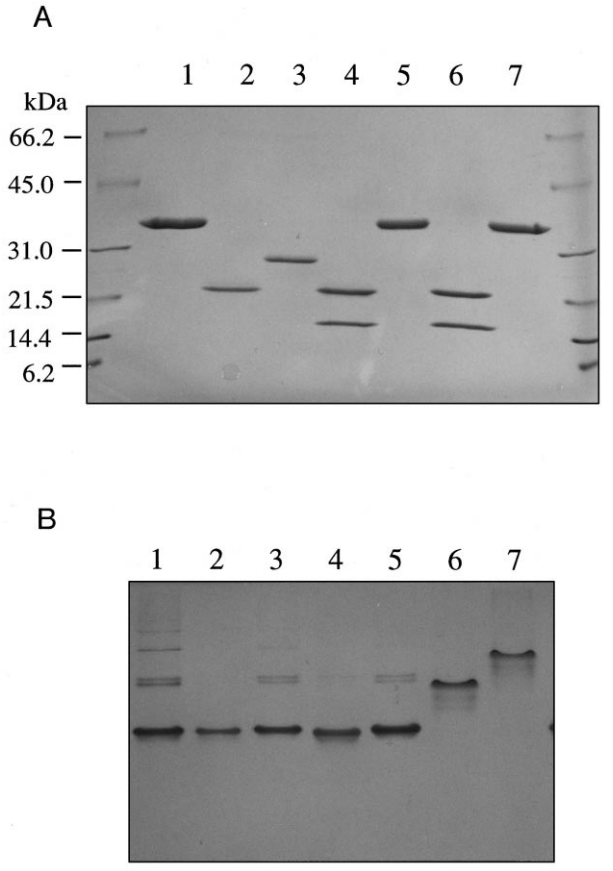

Fig. 5. Denaturing and non-denaturing polyacrylamide gel electrophoresis of mutant MSPs. A: SDS polyacrylamide gel electrophoresis. Denatured samples were applied to a 4–20% gradient polyacrylamide gel containing 0.1% SDS. Lane 1, wild-type MSP; lane 2, 156/end; lane 3, 189/end; lane 4, 156/M+157; lane 5, 156+M+157; lane 6, 156/L157M; lane 7, L157M. B: Non-denaturing polyacrylamide gel electrophoresis. Lane 1, wild-type MSP; lane 2, 156/M+157; lane 3, 156+M+157; lane 4, 156/L157M; lane 5, L157M; lane 6, 156/end; lane 7, 189/end.

We have not determined C-terminal sequences of the proteolytic products. To examine whether the loss of binding capacity was indeed caused by cleavage of the protein at a single site, we constructed two mutant MSPs that have a nick between Phe^{156} and Leu^{157}, a cleavage site specific to chymotrypsin. Because the mutant proteins had Met either before Leu^{157} (156/M+157) or in place of Leu^{157} (156/L157M), two non-nicked mutant proteins that have Met inserted between Phe^{156} and Leu^{157} (156+M+157) or in place of Leu^{157} (L157M) were constructed. A mutant (156/end) that corresponds to C2 was also constructed. Attempts to produce mutant MSPs with a nick between Arg^{189} and Phe^{190}, a trypsin-cleavage site, resulted in the expression of only the N-terminal fragment (189/end). As shown in Fig. 5A, all the mutant proteins migrated at expected positions of gels after denaturation. The two nick mutant proteins, 156/M+157 and 156/

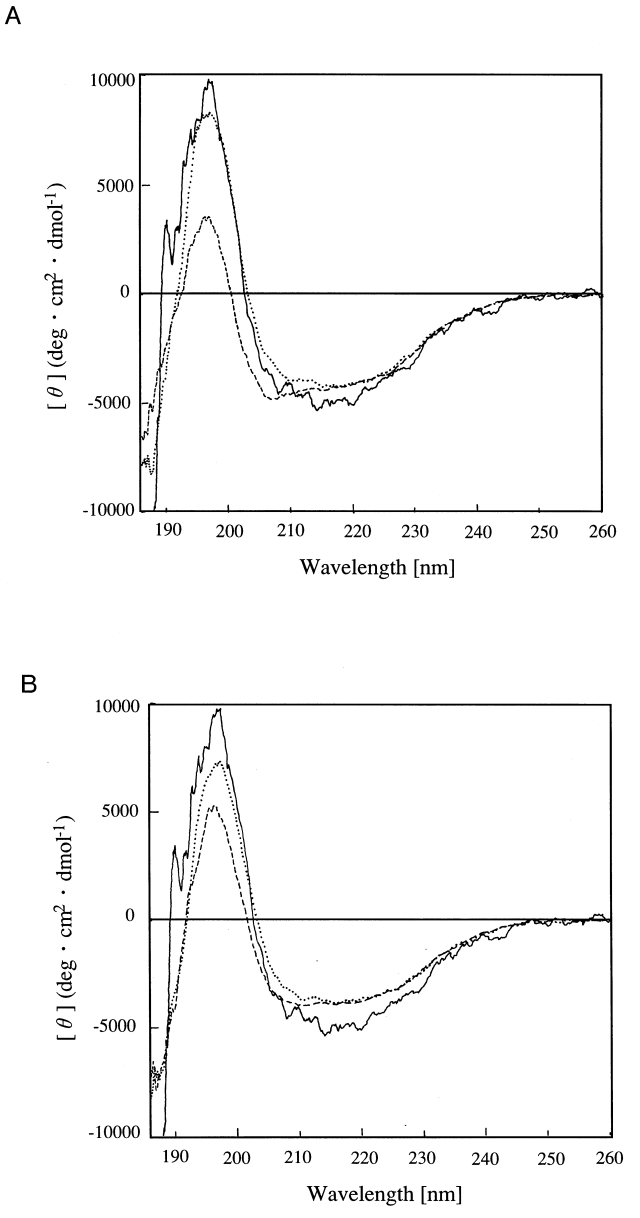

Fig. 6. CD spectra of mutant MSPs. A: Wild-type MSP (line); 156+M+157 (dotted line); 156/M+157 (dashed line). B: Wild-type MSP (line); L157M (dotted line); 156/L157M (dashed line). The spectra were scanned eight times (time constant, 0.5 s; sensitivity, 10 mdegree/FS; scan speed, 50 nm/min) and averaged.

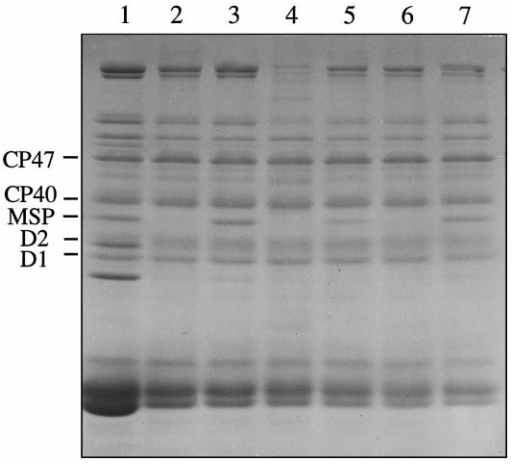

Fig. 7. Binding of mutant MSPs to PSII complexes. Experiments were carried out as in Fig. 3 except that urea/NaCl-washed PSII complexes were used in place of $CaCl_2$-washed PSII and a protein/PSII ratio of 5. A 12.5% polyacrylamide gel containing 0.1% SDS and 6 M urea was used. Lane 1, PSII complexes; lane 2, urea/NaCl-washed PSII complexes. Urea/NaCl-washed washed complexes were incubated with the wild-type MSP (lane 3), with 156/M+157 (lane 4), with 156+M+157 (lane 5), with 156/L157M (lane 6), and with L157M (lane 7).

L157M, yielded two fragments that showed electrophoretic mobilities similar to those of C2 and C3 (lanes 4 and 6). When subjected to non-denaturing gel electrophoresis, however, the two mutant proteins comigrated with the wild-type protein (Fig. 5B). This shows that introduction of a nick did not induce dissociation of the resulting polypeptide fragments. In other words, association and folding of the two polypeptide fragments spontaneously occurred when the mutant proteins were expressed in the bacterial cells.

The CD spectra of 156+M+157 and L157M were similar to that of the wild-type protein that is featured by a positive ellipticity at 197 nm and a negative ellipticity between 205 and 250 nm (Fig. 6). This indicates that the secondary conformation was little affected by insertion of Met between Phe^{156} and Leu^{157} or replacement of Leu^{157} by Met. The positive ellipticity was, however, appreciably reduced in both 156/M+157 and 156/L157M, suggesting that introduction of a nick resulted in a small but significant change in the protein conformation. The two N-terminal fragments, 156/end and 189/end were mostly in the random conformation (not shown).

Experiments were performed to examine binding capacity of the mutant proteins to urea/NaCl-washed PSII complexes (Fig. 7). The two mutant proteins with a nick showed no affinity to PSII complexes; none of their polypeptide fragments bound to urea/NaCl-washed PSII complexes (lanes 4 and 6). In contrast, 156+M+157 and L157M were found to bind to the PSII complexes (lanes 5 and 7). The inability of the nicked mutant proteins to bind the PSII complex is, therefore, related to introduction of a nick but not to insertion of or replacement by Met. The data presented in Table 3 show that L157M was effective in restoration of the oxygen-evolving activity of washed PSII complexes. Unexpectedly, however, the binding of 156+M+157 to PSII complexes was not associated with restoration of the oxygen-evolving activity. This finding led us to more detailed investigation of the binding of L157M and 156+M+157. Fig. 8 shows amounts of the mutant MSPs bound to urea/NaCl-washed PSII complexes as a function of protein to PSII ratio. The amount of the wild-type protein bound to PSII complexes at

Table 3
Oxygen-evolving activities of PSII complexes reconstituted with the mutant MSPs

Preparations	Additions	Oxygen evolution[a] (%)
PSII complexes	none	810 (100)
Urea/NaCl-washed PSII complexes	none	78 (10)
Urea/NaCl-washed PSII complexes	wild-type MSP[b]	410 (51)
Urea/NaCl-washed PSII complexes	156/M+157[b]	78 (10)
Urea/NaCl-washed PSII complexes	156+M+157[b]	95 (12)
Urea/NaCl-washed PSII complexes	156/L157M[b]	86 (11)
Urea/NaCl-washed PSII complexes	L157M[b]	452 (56)

[a] μmol O_2/mg chl per h.
[b] 5 mol MSP per mol PSII complexes.

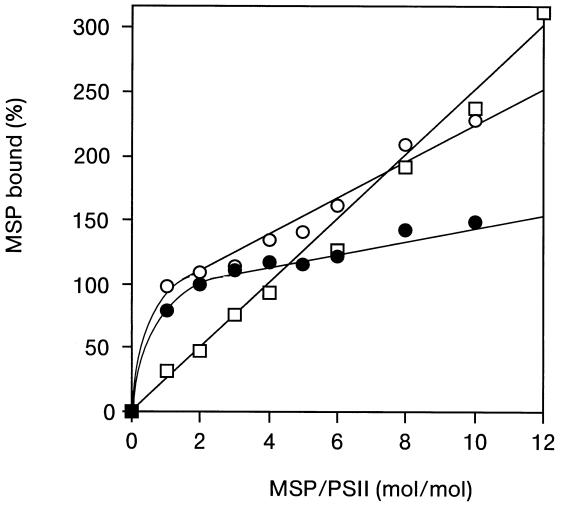

Fig. 8. Binding of L157M and 156+M+157 to urea/NaCl-washed PSII complexes. Each mutant protein was incubated with washed PSII complexes at indicated molar ratios of the protein to the PSII complex. Other experimental conditions were as in Fig. 3. 12.5% polyacrylamide gels containing 0.1% SDS and 6 M urea were used. Amounts of the mutant proteins bound to the complexes were determined by measuring peak area of the protein band relative to that of CP47, taking the amount of the wild-type protein bound to the complexes as 100%. Solids circles, wild-type MSP; open circles, L157M; open squares, 156+M+157.

a protein/PSII ratio of two was taken as 100%. L157M was able to bind to the PSII complex as strongly as the wild-type MSP and the high-affinity binding of both proteins was saturated at a protein/PSII ratio of one or two [27]. This, together with its ability to reactivate oxygen evolution, indicates that L157M can bind specifically to the functional site of the PSII complex. 156+M+157 bound to the PSII complex more weakly than L157M without showing saturation. Thus, amounts of 156+M+157 bound to PSII complexes exceeded that of the wild-type protein at high protein/PSII ratios. The result suggests that 156+M+157 bound to only non-specific sites of PSII complexes. L157M also showed a low affinity for non-specific sites, whereas the binding of the wild-type protein was highly specific to the functional site.

4. Discussion

The three proteases employed here primarily cleaved the *S. elongatus* MSP at two sites, and in spite of differences in the amino acid residue each protease recognizes, all the cleavage sites clustered between Phe^{156} and Gly^{163} and between Arg^{184} and Ser^{191}. This indicates that the two sequences are exposed on the outer surface of the protein. The secondary structure predicted from the multiple amino acid sequence alignment of MSPs from various organisms suggests that the Phe^{156}-Gly^{163} sequence is a part of a large loop structure and the Arg^{184}-Ser^{191} sequence is located in a turn structure between a helix and a β-sheet conformation [22]. Cleavage of the protein at a single site in one of the two regions resulted in the total loss of its binding capacity to PSII complexes. Proteolytic experiments suggest, therefore, that Phe^{156}-Gly^{163} and Arg^{184}-Ser^{191} are the regions for interaction with the PSII complex.

Site-directed mutagenesis provided evidence indicating that loss of the binding capacity is indeed caused by introduction of a nick in the two regions. The two mutant proteins with a nick between Phe^{156} and Leu^{157} (156/M+157 and 156/L157M) failed to bind to PSII complexes. This cannot be ascribed to dissociation or unfolding of the polypeptide fragments because the mutant proteins comigrated with the whole protein in non-denaturing gels. CD spectra showed, however, that the mutant MSPs had an altered secondary conformation compared with the wild-type protein. The result suggests that the loss of the binding capacity is related to a small structural disorder that was caused by cleavage of the protein.

The two mutant proteins that had no nick (156+M+157 and L157M) provided deeper insight into the structure-activity relationship of the cyanobacterial MSP. The CD spectra showed that the secondary structure of the protein was little affected by insertion of Met between Phe^{156} and Leu^{157} or by replacement of Leu^{157} by Met. L157M was able to bind specifically to the functional site of PSII complexes and reactivate oxygen evolution as effectively as the wild-type protein. Thus, Leu^{157} is not essential to the functional binding of the protein. In contrast to L157M, 156+M+157 bound only weakly and non-specifically to PSII complexes and failed to reactivate oxygen evolution. Thus, loss of the functional protein binding was not always associated with an appreciable change in the secondary conformation of the protein. Proteolytic and mutagenetic experiments

show that the capacity of the protein to bind to the PSII functional site is highly sensitive to a small change in the protein structure that is caused by cleavage of the protein or insertion of a single amino acid residue between Phe^{156} and Leu^{157}.

Interestingly, non-specific binding of the cyanobacterial MSP to the PSII complex was also influenced by a modification of the chymotrypsin-cleavage site. Binding to the PSII complex at sites other than the functional site was negligible with the wild-type protein but became appreciable by replacement of Leu^{157} by Met. Insertion of Met between Phe^{156} and Leu^{157} further increased binding of the protein to non-specific sites. We conclude, therefore, that the highly conserved Phe^{156}-Leu^{157} sequence is important for the protein to minimize non-specific binding to the PSII complex.

The Phe^{156}-Gly^{163} sequence is the largest amino acid sequence of MSP that is conserved among ten oxygenic photosynthetic organisms except for two cyanobacteria that have Ala in place of Gly^{161} (see Fig. 2). There are three charged residues, Asp^{158}, Lys^{160} and Arg^{162} in this sequence. The Arg^{184}-Ser^{191} sequence involves a highly conserved charged residue, Lys^{188}. As stated in Section 1, there is evidence suggesting that Asp^{157} and Lys^{159}-Lys^{186} of the spinach MSP, which correspond to Asp^{158} and Lys^{160}-Arg^{184} of the *S. elongatus* MSP, respectively, might be involved in the binding to the PSII complex [16,20]. Thus, the two regions studied here appear to be important for the binding of MSP to PSII complex in other photosynthetic organisms.

A different pattern of limited proteolysis was reported for the spinach MSP [15]. Chymotrypsin specifically cut the protein after Tyr^{16} and the resulting fragment of 24.6 kDa that had the C-terminal sequence identical to that of the intact protein failed to bind to PSII complexes. It was suggested, therefore, that the N-terminal sequence of the spinach MSP involves a site for interaction with the PSII complex. Chymotrypsin did not cut the cyanobacterial MSP near the N-terminus. This may reflect a situation that the cyanobacterial protein lacks a sequence Gln^{12}-Val^{19} that was implicated to involve a chymotrypsin-cleavage site in the spinach protein (see Fig. 6 of [15]). Recently, the Gln^{1}-Lys^{4} sequence of the spinach MSP was suggested to be a region for interaction with PSII complexes [16]. The corresponding sequence is not present in the N-terminus of the cyanobacterial MSP. It remains, therefore, to be investigated whether the N-terminal sequence is essential for the binding of the cyanobacterial MSP.

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