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Evidence that the amino-terminus of the 33 kDa extrinsic protein is required for binding to the Photosystem II complex

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Chymotrypsin and *Staphylococcus aureus* (strain V8) proteinase eliminated sixteen and eighteen amino acid residues, respectively, from the amino-terminal side of the extrinsic 33 kDa protein of the oxygen-evolving Photosystem II (PS II) complex of spinach. The carboxy-terminus of the resultant large fragments was found to be identical with that of the intact protein. Neither fragment could rebind to PS II membranes depleted of all the extrinsic proteins. Circular dichroism spectroscopy did not reveal any major conformational change within the two fragments. These results suggest that the amino-terminal region of the 33 kDa protein contains a domain essential for binding to the PS II complex.

Introduction

Photosystem II (PS II) catalyzes the light-dependent oxidation of water and the reduction of plastoquinone to plastoquinol in the thylakoid membrane. This pigment-protein complex is present in all oxygenic photosynthetic organisms and is composed of more than 15 polypeptides [1]. Detergent treatment of thylakoid membranes with Triton X-100 is able to produce thylakoid membrane sheets highly enriched in PS II that retain the ability to evolve oxygen in the presence of an artificial electron acceptor [2,3]. Biochemical studies with such PS II preparations together with inside-out PS II vesicles [4] demonstrated the presence of three extrinsic proteins, located on the luminal side of PS II, that participate in oxygen evolution (for a recent review see Ref. 1). Molecular masses for these proteins were estimated to be approx. 33, 23 and 18 kDa by SDS-polyacrylamide gel electrophoresis [5–7]. Subsequent analysis of the cDNAs for these proteins from spinach has revealed that the actual molecular masses are 26.7, 20.2 and 16.5 kDa, respectively [8,9].

The PS II complex contains one molecule each of the extrinsic proteins per reaction centre [10]. The 33 kDa protein stabilizes two of four Mn atoms present in the catalytic centre of oxygen evolution [11–13] and accelerates the S-state transition from S_3 to S_0 [14]. A discussion of the S states of the oxygen-evolving complex is given in Ref. 15. The 23 kDa protein participates in the trapping of Ca^{2+} in the intrinsic part of the PS II complex [1,16,17], and reduces the Cl^- requirement of oxygen evolution from 30 mM to 10 mM [18]. This protein also provides a binding site in the complex for the 18 kDa protein [19], which sustains oxygen evolution at Cl^- concentrations below 3 mM [18,20].

Partial degradation of the amino-terminal region of the 18 kDa and 23 kDa proteins suggested that this region contains a domain for binding to the PS II complex. Removal of twelve amino-acid residues from the amino-terminus of the 18 kDa protein by an endogenous proteinase eliminated the ability of this protein to rebind to PS II [21]. Digestion of nine amino-acid residues from the amino-terminus of the 23 kDa protein, by chymotrypsin, lowered the binding affinity of this protein while appearing not to significantly alter its conformation [22]. No such information is yet available for the 33 kDa protein.

The 33 kDa protein is the only extrinsic protein present in the PS II core complex prepared from PS II membranes [23,24], and it is necessary for full oxygen-evolving activity [25,26]. In addition, neither the 18 kDa nor the 23 kDa protein appears to be present in cyanobacteria [27]. However, studies in *Chlamydomonas reinhardtii* [28] and *Synechocystis* 6803 [29] have demonstrated that the 33 kDa protein is essential for stable oxygen evolution in vivo. In the present work we sub-

Abbreviations: Chl, chlorophyll; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; Mes, 4-morpholineethanesulphonic acid; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecylsulphate.

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jected purified 33 kDa protein to limited proteolysis using chymotrypsin and *Staphylococcus aureus* (strain V8) proteinase. This was done with a view to obtaining information regarding the functional domains of this protein. These proteolytic enzymes eliminated sixteen and eighteen amino-acid residues, respectively, from the amino-terminal region of the protein. The remaining large fragments could not rebind to PS II. Our results here support the conclusion that, as with the 18 kDa extrinsic protein, the amino-terminal region contains a domain required for binding to the PS II complex.

Materials and Methods

Preparation of PS II membranes

Photosystem II membranes were prepared from spinach (*Spinacia oleracea*) thylakoids with Triton X-100 according to the method of Kuwabara and Murata [3] and stored, in the presence of 30% (v/v) ethylene glycol, in liquid nitrogen. Tobacco (*Nicotiana sylvestris*) PS II membranes were prepared by the same method.

Purification of the 33 kDa protein

Photosystem II membranes were washed three times with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) (designated hereafter as medium A) by centrifugation at 35 000 × *g*, at 4° C for 10 min, and resuspension. All centrifugation steps below were performed at 4° C.

The 18 kDa and 23 kDa proteins were removed from PS II membranes by incubation with 1.0 M NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) at 1.0 mg Chl · ml^{-1} for 30 min, on ice, in the dark. The washed PS II were collected by centrifugation at 35 000 × *g* for 20 min. For complete removal of the 18 kDa and 23 kDa proteins, the membranes were washed once with 500 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5), collected by centrifugation at 35 000 × *g* for 10 min, and then washed and collected again in medium A.

The 33 kDa protein was extracted by incubating the NaCl-washed PS II membranes with 1.0 M $CaCl_2$/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) at 1.0 mg Chl · ml^{-1} for 30 min, on ice, in the dark. The extracted protein was separated from the membranes by centrifugation at 35 000 × *g* for 20 min. The supernatant was passed through a 5 μm filter (Acrodisc). For desalting the filtrate was applied to a Sephadex G-25 (Pharmacia) column (void volume 200 ml) equilibrated with 1.0 mM $(NH_4)_2SO_4$/10 mM Mes-NaOH (pH 6.5) at 4° C. The elution of the protein was detected at 280 nm. The conductivity of the sample was decreased to less than 1.0 mS · cm^{-1}.

The desalted protein solution was subjected to HPLC in an anion-exchange mode using a DEAE 650S column (2.2 cm i.d. × 20 cm, Tosoh) when more than 10 mg of 33 kDa protein were present or a DEAE 5PW column (0.75 cm i.d. × 7.5 cm, Tosoh) for amounts of less than 10 mg. In both cases the column was equilibrated with 1.0 mM $(NH_4)_2SO_4$/5.0 mM Mes-NaOH (pH 6.5) and eluted with a linear gradient from 1.0 to 50 mM $(NH_4)_2SO_4$ in 5.0 mM Mes-NaOH (pH 6.5) over 2 h, at a flow rate of 3.5 ml · min^{-1} (DEAE 650S) or 0.5 ml · in^{-1} (DEAE 5PW). The same method was used to prepare the 33 kDa protein from either spinach or tobacco PS II membranes.

Limited proteolysis of the spinach 33 kDa protein

A chymotrypsin fragment was obtained by incubating the purified spinach 33 kDa protein with α-chymotrypsin (Type VII from bovine pancreas, Sigma) which had been treated with 1-chloro-3-tosylamide-7-amino-heptanone to inactivate trypsin. The reaction mixture contained 0.1 mM $CaCl_2$/5.0 mM Mes-NaOH (pH 6.5) with the protein to enzyme ratio fixed at 100 : 1 (mol/mol). The incubation was for 30 min at 20° C and the reaction was stopped by adding PMSF to a final concentration of 3.0 mM. The 33 kDa protein was also digested with *S. aureus* (strain V8) proteinase (Type XVII, Sigma) in a fashion identical to that used with chymotrypsin but with $CaCl_2$ and PMSF omitted. In addition, the incubation time was extended to 90 min.

The fragments were recovered by HPLC in an anion-exchange mode (DEAE 5PW) as described above for the purification of the 33 kDa protein. The purified fragment was then dialysed against 10 mM Mes-NaOH (pH 6.5) using 15 000 molecular-weight cut-off wet tubing (Spectrum). The fragments were stored at −80° C until use. Reconstitution experiments (see below) were performed with both dialysed and undialysed fragments to eliminate the possibility of any conformational change, introduced during the dialysis step, contributing to the observed results.

The concentration of the 33 kDa protein was determined using a molar absorption coefficient of 16 $mM^{-1} \cdot cm^{-1}$ at 276 nm. This value was obtained from that previously reported [30] with correction for the molecular mass of 26.7 kDa determined from the amino-acid sequence of this protein [31]. The concentrations of the fragments obtained by treatment with the proteinases were determined using a molar absorption coefficient of 14 $mM^{-1} \cdot cm^{-1}$ at 276 nm which was calculated from the number of tyrosine, tryptophan and cysteine residues in these fragments [32].

Reconstitution with the 33 kDa protein and its fragments

To prepare PS II membranes for the reconstitution experiments, untreated spinach PS II membranes were incubated at 0.5 mg Chl · ml^{-1} with 1.0 M NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) for 30 min on ice, in the dark, and then collected by centrifugation at 35 000 × *g* for 20 min. The pellet was resuspended

and washed in 200 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) (designated hereafter as medium B) and collected by centrifugation at 35 000 × *g* for 10 min. The PS II membranes were then resuspended in medium B at 3.0 mg Chl · ml^{-1} and adapted to darkness, on ice, for 60 min. To these membranes 5 vol. of 3.1 M urea/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) were added to give a final concentration of 2.6 M urea and 0.5 mg Chl · ml^{-1}. The membranes were then incubated for 30 min on ice, in the dark, and then collected at 35 000 × *g* for 20 min. The pellet was then washed twice in medium B by resuspension and centrifugation at 35 000 × *g* for 10 min and finally suspended in medium B at 3.0 mg Chl · ml^{-1}, after which the membranes were stored in liquid nitrogen until use.

Rebinding studies were performed in 1.0 ml aliquots of medium A to which the NaCl/urea-treated PS II membranes had been added at 100 μg Chl · ml^{-1}. When undialysed fragments were used 15 mM $(NH_4)_2SO_4$ was present. The 33 kDa protein or fragments were added to give the desired protein-to-chlorophyll ratios (see Results and Discussion and figure legends). The rebinding was allowed to take place in the dark, on ice, for 30 min and the membranes were then collected at 35 000 × *g* for 20 min using a Beckman TL-100 ultracentrifuge. The membranes were then washed twice in medium A by resuspension and centrifugation at 35 000 × *g* for 20 min to ensure removal of all unbound protein. The membranes were finally resuspended in medium A at 1.0 mg Chl · ml^{-1} and subjected to SDS-polyacrylamide gel electrophoresis using the system of Laemmli [33]. The polyacrylamide concentrations of the stacking and separation gels were 5% and 12%, respectively. The urea concentration was 6.0 M. The gels were stained with Coomassie brilliant blue R-250 and the electrophoretic patterns were photographed or recorded with a dual-wavelength TLC scanner (CS 930, Shimadzu).

Oxygen-evolution activity was measured as described in Kuwabara et al. [13]. In the present study the reaction medium contained 10 mM NaCl/10 mM $CaCl_2$/300 mM sucrose/25 mM Mes-NaOH (pH 6.5)/0.3 mM phenyl-*p*-benzoquinone, with a chlorophyll concentration of 8 μg Chl · ml^{-1}, and the 33 kDa protein or fragments were added to give the required protein-to-chlorophyll ratios. When undialysed fragments were used 3.5 mM $(NH_4)_2SO_4$ was present. The chlorophyll concentration was determined according to Arnon [34].

Partial amino-acid sequences

The amino-terminal amino-acid sequences of the 33 kDa protein and the fragments derived by limited proteolysis were determined according to Matsudaira [35] as modified by Murata et al. [36] employing a gas-phase protein sequence analyzer (470A, Applied Biosystems). The sequence at the carboxy-terminus was examined by analyzing the amino acids released by carboxypeptidase digestion with an amino-acid analyzer (835, Hitachi) equipped with a fluorometric detection system as described in Ref. 22.

Circular dichroism

Measurements of circular dichroism spectra were carried out with a spectropolarimeter (J-40A, JEOL), equipped with a data processor (J-DPZ, JEOL). The conditions of measurement were: 0.1 cm light path length; 4 s time constant; 2 millidegree · cm^{-1} sensitivity; 5 ms sampling time; 5 nm · min^{-1} scan speed; and 20 °C temperature. Sixteen scanning spectra were accumulated for averaging. Additional details are provided in the legend to Fig. 5.

Results and Discussion

Purification and characterization of proteolysis fragments from the spinach 33 kDa protein

The final HPLC purification step for the extrinsic spinach 33 kDa protein is shown in Fig. 1a. The protein eluted at approx. 20 mM $(NH_4)_2SO_4$ as a prominent

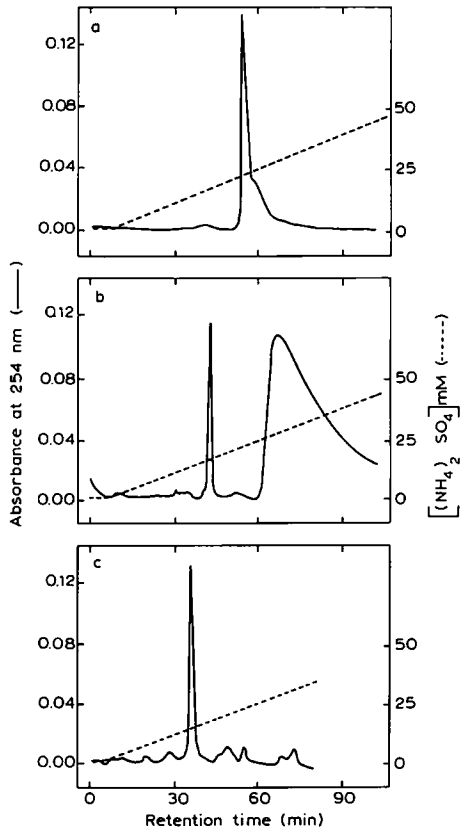

Fig. 1. HPLC of the spinach 33 kDa protein and its proteolysates produced by chymotrypsin and *S. aureus* (strain V8) proteinase. The conditions of measurement were: 0.5 ml min^{-1} flow rate, 10 °C temperature, and the column was a DEAE 5PW (0.75 cm i.d. × 7.5 cm, Tosoh). (a) 33 kDa protein extract (approx. 3 mg); (b) proteolysate of the 33 kDa protein (1.4 mg) produced with chymotrypsin and (c) proteolysate of the 33 kDa protein (1.4 mg) produced with *S. aureus* (strain V8) proteinase.

peak with a minor shoulder. Subsequent application of either the shoulder or the major peak component to a second HPLC step again yielded a single peak with a shoulder in each case as shown in Fig. 1a. This may suggest that the characteristic shape reflects an equilibrium between two conformational states of the protein which bind to the anion-exchange resin with slightly differing affinities. A small peak, eluting at approx. 14 mM $(NH_4)_2SO_4$, was due to contaminating 23 kDa extrinsic protein that had not been completely removed by the NaCl wash. The 33 kDa extrinsic protein purified in this way was then incubated with chymotrypsin or *Staphylococcus aureus* (strain V8) proteinase and applied to the HPLC column.

The protein fragment produced with chymotrypsin (hereafter referred to as the CH fragment) eluted at approx. 16 mM $(NH_4)_2SO_4$ (Fig. 1b). The large broad peak eluting after 25 mM $(NH_4)_2SO_4$ was due to PMSF which was added to terminate the proteolysis. The fragment produced with *S. aureus* (strain V8) proteinase (hereafter referred to as the V8 fragment) eluted at approx. 12 mM $(NH_4)_2SO_4$ (Fig. 1c). The remaining peaks in the chromatogram for Fig. 1c were additional degradation products following proteolysis of the intact protein. Application of either chymotrypsin or *S. aureus* (strain V8) proteinase to the HPLC column demonstrated that these enzymes eluted at 12 mM $(NH_4)_2SO_4$ and 9 mM $(NH_4)_2SO_4$, respectively (data not shown). The yield for both the CH and V8 fragments was typically 15%.

The amino-terminal sequences for the intact 33 kDa protein and both the CH and V8 fragments are presented in Fig. 2. The results show that chymotrypsin cut the 33 kDa protein at the carboxy-side of tyrosine-16 and *S. aureus* (strain V8) proteinase cut at the carboxy-side of glutamate-18. The sequences at the carboxy-terminus for both fragments are also shown in Fig. 2 and were identical to that of the intact protein. The relative molecular masses of the CH and V8 fragments are calculated to be 24.6 and 24.4 kDa, respectively. However, in the case of the V8 fragment we were unable to determine unequivocally whether glutamine-247 was present. Physicochemical properties of the 33 kDa protein and the two fragments are compared in Table I. The removal of glutamate-18 by *S. aureus* (strain V8) proteinase is accompanied by a small change in the isoelectric point from 5.2 to 5.3.

TABLE I

Properties of the spinach 33 kDa extrinsic protein and the two fragments produced by chymotrypsin (CH) and S. aureus (strain V8) proteinase (V8)

n.d., not determined.

Protein or fragment	Molecular weight	Isoelectric point	ε at λ_{max} ($mM^{-1} \cdot cm^{-1}$)	
			measured	calculated [a]
33 kDa	26533	5.2	16	16.7
CH fragment	24663	5.2	n.d.	14.0
V8 fragment	24421	5.3	n.d.	14.0

[a] See Ref. 32.

Reconstitution studies with the CH and V8 fragments of the spinach 33 kDa protein

Fig. 3 presents the oxygen-evolution activity of the NaCl/urea-washed PS II membranes when the CH and V8 fragments were added. Trace (a) in Fig. 3 is from NaCl/urea-washed membranes and shows no oxygen-evolution activity. The CH fragment was unable to support oxygen evolution in trace (b) at a protein-to-chlorophyll ratio of 0.6. At a protein-to-chlorophyll ratio of 3.0 neither the CH nor the V8 fragment was able to sustain oxygen evolution (traces (c) and (d)). This contrasts with trace (e), where the 33 kDa protein, at a protein-to-chlorophyll ratio of 0.6, reconstituted oxygen evolution at 250 μmol O_2/mg Chl per h. Oxygen evolution in the intact system is shown in trace (f).

The rebinding of the CH and V8 fragments was investigated in the experiment shown in Fig. 4. Figure 4a–c demonstrates that at a protein-to-chlorophyll ratio of 0.6 in the reaction mixture, rebinding of the 33 kDa protein at the original level was obtained. As with the oxygen-evolution activity measurements, rebinding of the 33 kDa protein to NaCl/urea-washed PS II is expected to saturate at a protein-to-chlorophyll ratio of approx. 0.3 [13].

By adding measured amounts of the purified fragments to gel lanes containing NaCl/urea-washed PS II membranes the purified fragments served as markers for the location of the fragments in the densitograms. Fig. 4d shows 1.0 μg of the CH fragment added as a marker. In the case of the CH fragment, Fig. 4d–f demonstrates that, at a protein-to-chlorophyll ratio of 0.6, virtually no rebinding could be detected in the densitogram. Similarly, at a protein-to-chlorophyll ratio

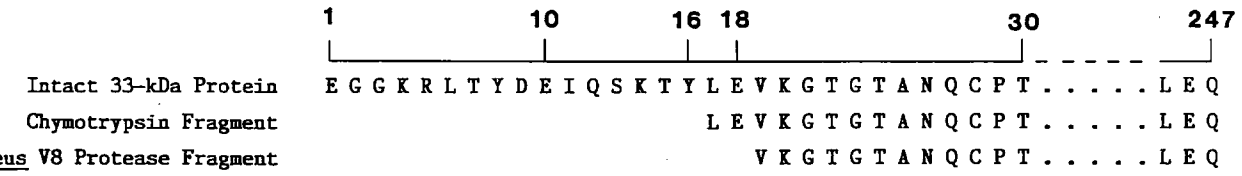

Fig. 2. The amino-terminal amino-acid sequence and the carboxy-terminus for the intact spinach 33 kDa protein, the fragment produced with chymotrypsin and the fragment produced with *S. aureus* (strain V8) proteinase.

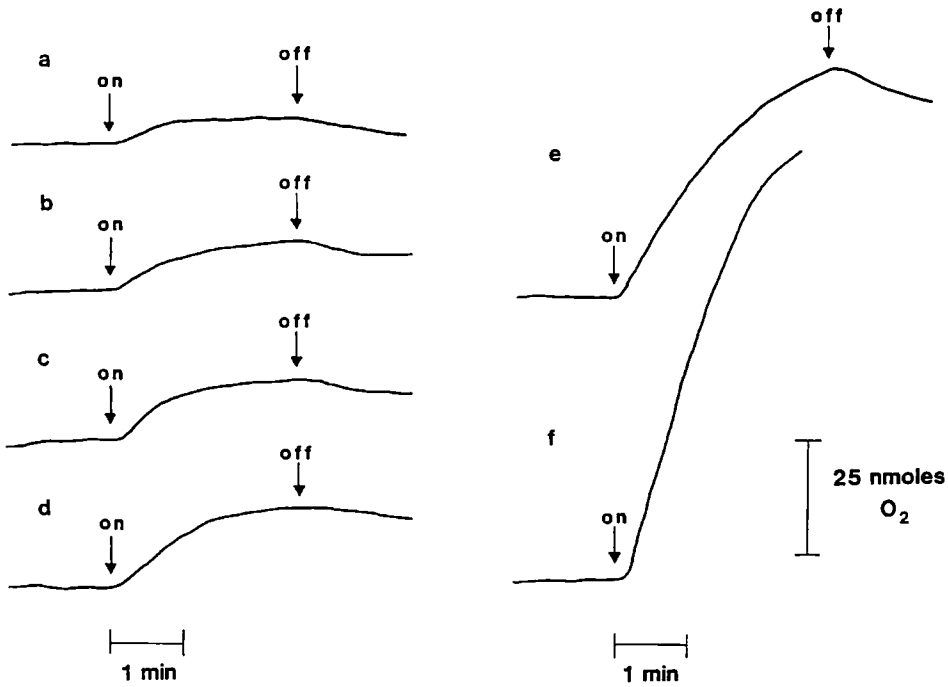

Fig. 3. Oxygen evolution in spinach PS II membranes depleted of the 33 kDa extrinsic protein and supplemented with fragments produced by chymotrypsin (CH) and *S. aureus* (strain V8) proteinase (V8). (a) NaCl/urea-washed PS II membranes; (b) NaCl/urea-washed PS II membranes reconstituted with the CH fragment at a protein-to-chlorophyll ratio 0.6 (w/w), or a protein to reaction centre ratio of 5 (mol/mol); (c) as (b) but with the CH fragment at a protein-to-chlorophyll ratio of 3.0 (w/w), or a protein to PS II reaction centre ratio of 24 (mol/mol); (d) as (b) but with the V8 fragment at a protein-to-chlorophyll ratio of 3.0 (w/w), or a protein to PS II reaction centre ratio of 24 (mol/mol); (e) NaCl/urea-washed PS II membranes reconstituted with the 33 kDa protein at a protein-to-chlorophyll ratio of 0.6 (w/w), or a protein to reaction centre ratio of 5.0 (mol/mol); and (f) untreated PS II membranes. The initial rates of oxygen evolution activity in traces (e) and (f) were 250 and 480 μmol O_2/mg Chl per h, respectively. The reaction medium contained 10 mM NaCl/10 mM $CaCl_2$/300 mM sucrose/25 mM Mes-NaOH (pH 6.5)/0.3 mM phenyl-*p*-benzoquinone and NaCl/urea-washed PS II membranes corresponding to 8 μg Chl·ml^{-1}. Undialysed CH and V8 fragments were used and therefore 3.5 mM $(NH_4)_2SO_4$ was also present (see Materials and Methods). Identical results were obtained with dialysed fragments (data not shown; cf. Fig. 4). In calculating the molar ratio of protein to reaction centre, the Chl to PS II reaction centre ratio was taken as 220:1 (mol/mol) [10]. Arrows indicate when the light was turned on or off.

of 3.0 virtually no rebinding of the CH or V8 fragment could be measured (data not shown).

During the preparation of the CH fragment PMSF was added to terminate chymotrypsin activity. This proteinase inhibitor is known to specifically modify serine residues [37]. Therefore, a control experiment was conducted where PMSF was added but chymotrypsin omitted during the incubation. No effect on rebinding of the 33 kDa protein was detected after a 30 min incubation with 3.0 mM PMSF at 10°C (data not shown). This result suggests that the inability of the CH fragment to rebind to the PS II complex was not caused by the effect of PMSF.

Circular dichroism

Measurements of circular dichroism spectra for proteins in the ultraviolet region provide a sensitive monitor of conformational changes. The effect of removing sixteen or eighteen amino acids from the amino-terminal region of the 33 kDa protein was investigated by circular dichroism and is shown in Fig. 5. This technique has previously been applied to the 33 kDa protein by Tanaka and Wada [38] who showed that reduction with mercaptoethanol of the disulphide bond between cysteine-28 and cysteine-51 of the spinach 33 kDa protein produced a deep negative molar ellipticity below 220 nm characteristic of a random conformation. The circular dichroism spectrum for the intact 33 kDa protein, shown in Fig. 5a, possessed a negative molar ellipticity between 250 and 220 nm and a large positive molar ellipticity at approx. 210 nm.

The circular dichroism spectrum for the V8 fragment is presented in Fig. 5b. The spectrum for this fragment is almost identical with that shown in Fig. 5a for the intact protein. This suggests that, despite the inability of this fragment to rebind to the PS II complex, the conformation of the fragment remained essentially unchanged. An identical result was obtained with the larger CH fragment (data not shown).

These data suggest that the removal of sixteen or eighteen amino-acid residues from the amino-terminus of the 33 kDa protein specifically eliminates the capac-

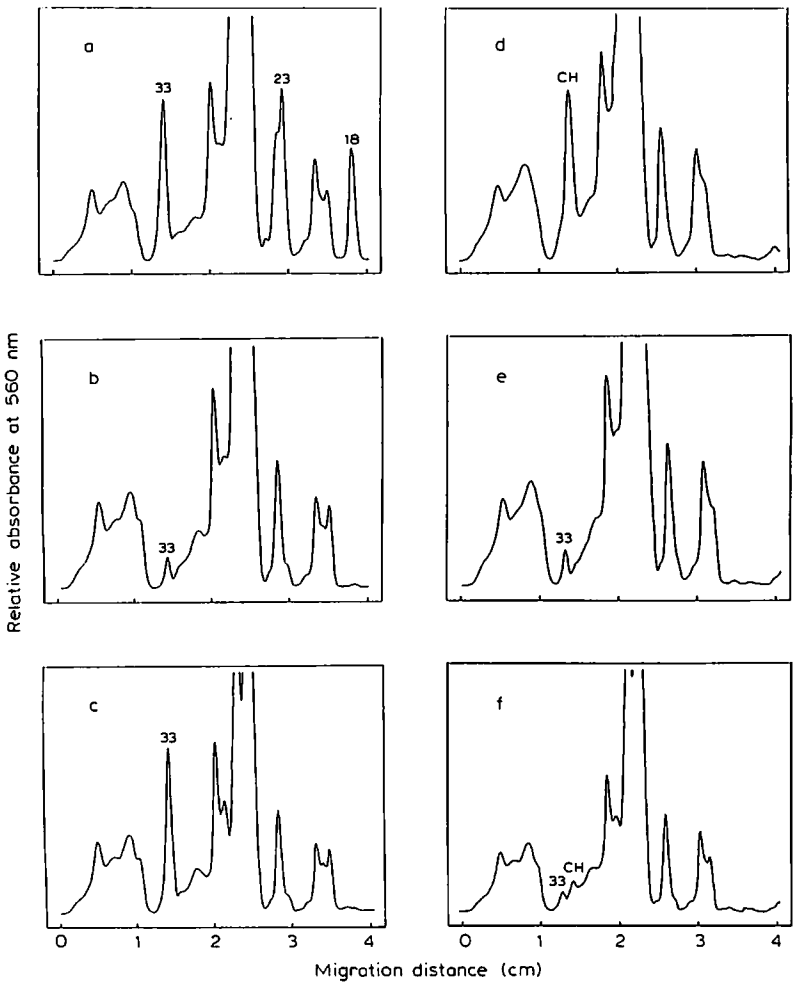

Fig. 4. Densitograms of spinach PS II proteins: (a) PS II membranes; (b) NaCl/urea-washed PS II membranes; (c) NaCl/urea-washed PS I membranes reconstituted with the 33 kDa protein at a protein-to-chlorophyll ratio of 0.6 (w/w), or a protein to PS II reaction centre ratio of : (mol/mol); (d) mixture of NaCl/urea-washed PS II membranes and the chymotrypsin fragment added as a marker; (e) NaCl/urea-washed PS II membranes; (f) NaCl/urea-washed PS II membranes reconstituted with the chymotrypsin-derived fragment at a protein-to-chlorophyll ratio of 0.((w/w), or a protein to PS II reaction centre ratio of 5 (mol/mol). The experiment shown employed dialysed fragments. Identical results were obtained with undialysed fragments (data not shown; cf. Fig. 3). The Chl to PS II reaction centre ratio was taken as 220 : 1 (mol/mol) [10].

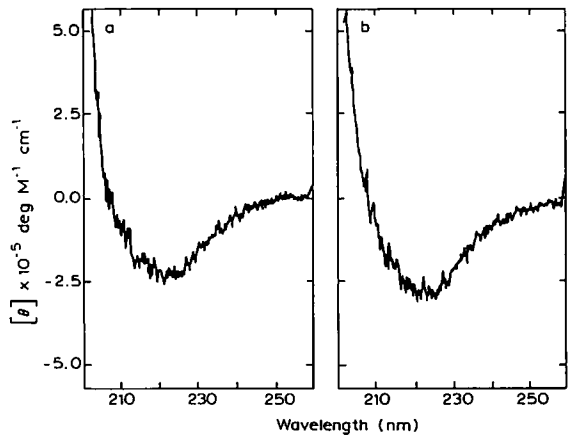

Fig. 5. Circular dichroism spectra of spinach 33 kDa protein and its fragment in the ultraviolet region: (a), the intact 33 kDa protein; and (b) the *S. aureus* (strain V8) proteinase-derived fragment. The protein was dissolved in 10 mM Mes-NaOH (pH 6.5) at a concentration of 6 μM.

ity of this protein to rebind to PS II complex without inducing a major conformational change throughout the protein.

The binding domain of the 33 kDa protein

Fig. 6 presents the amino-terminal sequences from five known 33 kDa proteins. The numbering for the spinach sequence is shown. New data for the amino-terminal sequence of the 33 kDa protein from tobacco are presented. This region of the protein is 86% homologous with spinach and 89% homologous with pea, based on a comparison of the amino-acid residues, and a number of the substitutions between spinach, pea and tobacco are rather conservative. Glycine-3 in spinach becomes alanine in pea and valine in tobacco. Phenylalanine in pea and tobacco is changed to tyrosine at position 8 in spinach thus preserving the aromatic side-chain. Valine-31 in spinach replaces isoleucine in

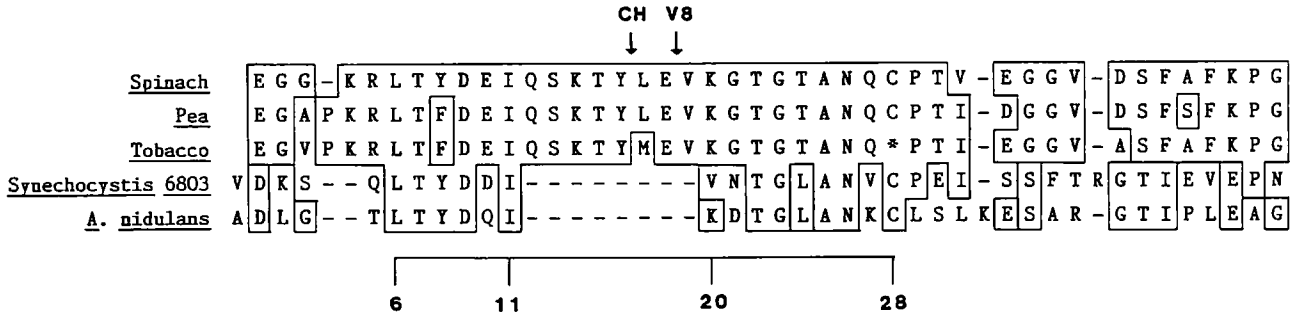

Fig. 6. The amino-terminal amino-acid sequence of the 33 kDa extrinsic protein from five different species. Arrows show the cutting sites for chymotrypsin (CH) and *S. aureus* (strain V8) proteinase (V8) in the spinach sequence. The solid line indicates a possible functional domain suggested by Kuwabara et al. [39]. The sequence data are from: spinach [31]; pea [40]; tobacco (the present study); *Synechocystis* PCC6803 [29]; and *A. nidulans* [39].

pea and tobacco, shortening the aliphatic side-chain by one carbon, and alanine-39, present in both spinach and tobacco, is changed to serine in pea. Similarly, the substitution of aspartate in pea at position 32 for glutamate in spinach and tobacco may be deemed conservative, since this maintains the presence of an acidic side-chain. The two remaining substitutions are more striking. Leucine-17 in spinach and pea is changed to methionine in tobacco, thus introducing a sulphur-containing side-chain at this position, while aspartate-36 in spinach and pea becomes alanine in tobacco, suggesting that a charged carboxyl group at this position is not essential. The last remaining variation is the deletion of a proline residue between glycine-3 and lysine-4 in the spinach sequence. However, it must be concluded from these sequence data that the amino-terminal region for higher plants shown in Fig. 6 is highly conserved.

Kuwabara et al. [39] have identified five putative functional domains from a sequence comparison of *Anacystis nidulans* with spinach and pea 33 kDa proteins [31,39,40]. This assignment is based on five regions of relatively high homology, even though the overall sequence homology between *A. nidulans,* spinach and pea is only 48–49%. As shown in Fig. 6 the CH and V8 fragments arise from cutting sites in one of the conserved domains suggested by Kuwabara et al. [39]. It is therefore possible that the domain indicated in Fig. 6 is an essential component of the binding domain for the PS II complex. Since a deletion is evident in the *A. nidulans* sequence between isoleucine-11 and lysine-20, the sequence from leucine-6 to isoleucine-11 may constitute the important region for binding.

Philbrick and Zilinskas [29] have reported the sequence of the extrinsic 33 kDa protein from *Synechocystis* PCC6803. Fig. 6 also shows that the conserved region from leucine-6 to isoleucine-11 is also present in this sequence when an identical deletion, as suggested for *A. nidulans,* is assumed to exist between isoleucine-11 and lysine-20. Therefore, the sequence Leu(6)-Thr(7)-Tyr/ Phe(8)-Asp(9)-Glu/ Asp(10)-Ileu(11) would appear to be required either for maintaining the tertiary structure of the binding domain or for binding to the PS II complex directly. However, in the case of *A. nidulans* glutamate-10 is changed to glutamine, the uncharged derivative of glutamate, suggesting that the inclusion of a charged residue at this position is not as critical for binding as aspartate at position 9 or the presence of an aromatic side-chain at position 8.

Conclusion

Incubation of the purified extrinsic 33 kDa of spinach PS II with chymotrypsin or *Staphylococcus aureus* (strain V8) proteinase produced two large fragments. Both fragments could be recovered and purified by HPLC. The chymotrypsin-derived fragment was cut at the carboxy-side of tyrosine-16 and the *S. aureus* (strain V8) proteinase-derived fragment was cut at the carboxy-side of glutamate-18. The carboxy-terminus of both fragments was identical to that of the intact protein. Neither of the fragments possessed the ability to rebind to NaCl/urea-washed PS II membranes and accordingly neither fragment could reconstitute oxygen-evolution activity. Circular dichroism spectra for the two fragments did not reveal any major conformational change. The amino-terminal cutting sites also fell within a domain of high sequence homology between the *A. nidulans, Synechocystis* PCC6803, spinach, pea and tobacco 33 kDa extrinsic proteins. In particular, the region leucine-6 to isoleucine-11 in the spinach sequence, within this domain, is conserved in all 33 kDa protein sequences so far reported and we suggest that this could be a critical region for binding to the PS II complex.

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