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Studies on the polypeptide composition of the cyanobacterial oxygen-evolving complex

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Various approaches have been used to investigate the polypeptides required for oxygen evolution in cyanobacteria, in particular the thermophile *Phormidium laminosum*. Antibodies against the extrinsic 33 kDa protein from spinach Photosystem II cross-reacted clearly in immunoblotting experiments with a corresponding polypeptide in isolated thylakoids and Photosystem II particles from *P. laminosum* and with whole-cell homogenates of three species of cyanobacteria (*Phormidium laminosum*, *Synechococcus leopoliensis* and *Anabaena variabilis*). In contrast, no cyanobacterial proteins reacted with antibodies against the 23 and 16 kDa proteins of spinach Photosystem II. The lack of cross-reactivity and the absence of these polypeptides from highly active Photosystem II particles of *Phormidium laminosum* strongly suggest that cyanobacteria do not contain polypeptides corresponding to these two chloroplast proteins. Treatment of *P. laminosum* Photosystem II particles with 0.8 M alkaline Tris, 1 M NaCl, $CaCl_2$ or $MgCl_2$ inhibited O_2 evolution, and quantitatively removed a 9 kDa polypeptide from the particles. None of these treatments removed comparable amounts of the 33 kDa polypeptide, and only Tris treatment removed manganese. The release of the 9 kDa polypeptide upon NaCl treatment correlated well with the deactivation at the donor side of Photosystem II. A direct connection between the 33 kDa polypeptide and O_2 evolution was established by the finding that trypsin treatment digested this polypeptide and inhibited O_2 evolution in parallel.

Introduction

Numerous studies (reviewed in Ref. 1) have identified three proteins of approx. 33, 23 and 16 kDa, which participate in reactions on the oxidizing side of Photosystem II in higher plants. All three are extrinsic proteins attached to the inner surface of the thylakoid membrane, from which they can be removed by treatments such as washing with 0.8 M alkaline Tris, 1 M $CaCl_2$ or $MgCl_2$ [1,2]. In a recent report, a fourth polypeptide, of 10 kDa, has also been implicated in this process [3], due to its release by Tris from the inner thylakoid surface. The 33 kDa protein can be isolated with manganese bound to it under some conditions [4,5], and may play a role in binding or stabilizing manganese at the catalytic centre of the oxygen-evolving complex [6,7]. The 23 kDa and 16 kDa proteins are involved in mediating high-affinity binding of the essential chloride ions to the complex [8–10]. One or both of these proteins may

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; LiDS, lithium dodecyl sulphate; Mes, 4-morpholineethanesulphonic acid.

also promote calcium binding around the oxygen-evolving site [10–12]. Very recent reports [6,7] suggest that calcium and chloride ions may also be implicated in the function of the 33 kDa protein. So far, it has not been possible to assign any definite role to the 10 kDa polypeptide.

In contrast to the wealth of studies on higher plants, there is less information on the proteins participating in oxygen evolution in the only free-living prokaryotes capable of oxygenic photosynthesis, the cyanobacteria. However, studies on the active oxygen-evolving Photosystem II preparations which have now been isolated from a number of cyanobacterial species [13–17] are likely to rectify this situation. In the present study an improved preparation of both highly active and substantially purified Photosystem II oxygen-evolving particles from *Phormidium laminosum* [18,19] has been used to identify specifically polypeptides functioning on the water-oxidation side of Photosystem II. To this end, we have used two separate approaches. Firstly, we have investigated whether cyanobacteria contain proteins corresponding to the spinach 33, 23 and 16 kDa proteins. This was done by SDS-polyacrylamide gel electrophoresis of various cyanobacterial cells, thylakoids and Photosystem II particles in combination with western blotting using antibodies against each of the three spinach proteins. Secondly, we have applied to Photosystem II particles from *P. laminosum* some of the treatments used to inhibit oxygen evolution and remove or modify specific polypeptides from thylakoids of higher plants. These approaches have enabled us to identify two polypeptides, 33 and 9 kDa, which function on the oxidizing side of Photosystem II in *P. laminosum*, but our results give no evidence for the presence of 23 and 16 kDa proteins equivalent to those of higher-plant Photosystem II.

Materials and Methods

P. laminosum spheroplasts, thylakoids and Photosystem II particles were prepared as described previously [19,20]. Before use, the Photosystem II particles were concentrated to 0.1 mg Chl/ml in an Amicon ultrafiltration cell (100 000 kDa cut-off membrane).

Synechococcus leopoliensis (CCAP 1405/1, also called *Anacystis nidulans*) and *Anabaena variabilis* (CCAP 1403/12) were grown at 25°C in Medium BG-11 of Stanier et al. [21] with other conditions as in Ref. 20. Cells were treated at 30°C for 4 h with 0.1% lysozyme in a buffer containing 0.5 M sorbitol, 30 mM $CaCl_2$, 10 mM Hepes/NaOH (pH 7.2) and 12.5 mM EDTA. The cells were then diluted with cold buffer (minus EDTA), spun down, washed once and then resuspended in the same buffer, then broken by sonication (six 30 s bursts at maximum power using a 1 cm diameter probe, cooling on ice between bursts). *A. variabilis* cells were broken by a similar sonication procedure without prior lysozyme treatment. For both cyanobacteria, samples of the total cell homogenate were used for electrophoresis. For cyanobacteria, Chl *a* was assayed by the method of Arnon et al. [22]

Photosystem II particles were prepared from spinach thylakoids as in Refs. 23 and 24 using Triton X-100 extraction. Tris-washing was performed by incubation of the Photosystem II particles at 0.5 mg Chl/ml in 0.8 M Tris-HCl (pH 8.4) on ice in room light for 30 min, followed by centrifugation at 100 000 × *g* for 30 min. A simple wash procedure could not be used for treatment of Photosystem II particles from *P. laminosum* with Tris or 1 M salts, because there was general proteolytic degradation of the particles, even in the presence of protease inhibitors, during the long centrifugation time required (15 h). Therefore, a chromatographic method taking approx. 2 h was devised for the treatments. 0.9 ml samples of particles were diluted to 1.2 ml with concentrated stock solution to give the final desired concentration of Tris (0.8 M, pH 8.5) or NaCl, $MgCl_2$ or $CaCl_2$ (0.2–1.0 M, pH 6.5). The samples were incubated on ice for 15 min, then applied to 1.5 × 9.5 cm columns of Sephacryl S-300 equilibrated with buffer containing 20% (v/v) glycerol/10 mM $MgCl_2$/40 mM Mes-NaOH (pH 6.5) and Tris or salt at the same final concentration. The eluted green particles (2.3 ml) were followed by two pale blue fractions (each 2.3 ml). All fractions were desalted by passage through a 1.5 × 10 cm column of Sephadex G-25 (equilibrated with 20% glycerol/10 mM $MgCl_2$/40 mM Mes, pH 6.5) and then concentrated to 0.5 ml by ultrafiltration in an Amicon M3 cell (XM100A mem-

brane). In the case of $CaCl_2$ treatment, some irreversible adsorption to the Sephacryl gel lowered the recovery of the treated particles.

For trypsin treatment, samples of Photosystem II particles (20 μl) were mixed with 20 μl of 50 mM Tricine-NaOH (pH 7.5), containing the desired concentration of enzyme (Sigma, Type XI). The samples were incubated at 25°C for 3 min, then the reaction stopped either by addition of 20 μg trypsin inhibitor (Sigma, Type II-0) for oxygen evolution assays, or 20 μl of hot (60°C) solubilising buffer for polyacrylamide gel electrophoresis (8% LiDS/0.01% bromophenol blue/20% glycerol/0.2 M Tris-HCl, pH 6.8). A control in which solubilizing buffer was added before the maximum amount of trypsin showed that the hot solubilizing buffer did stop the reaction.

Oxygen evolution was assayed at 25°C in an oxygen electrode, at saturating intensities of red light. The assay mixtures contained 40 mM Mes-NaOH (pH 6.5) or 40 mM Hepes-NaOH, (pH 7.2 or 7.5) plus 25% (v/v) glycerol, salts as detailed in the individual Figure legends, and Photosystem II particles equivalent to 2–3 μg Chl *a* in a final volume of 1.0 ml. Electron acceptors were 1 mM phenyl-*p*-benzoquinone or 10 mM ferricyanide. DCMU, when used, was at 10 μM. Fluorescence induction measurements were made in 10 mM Hepes-NaOH/5 mM sodium phosphate buffer (pH 7.5)/10 mM $MgCl_2$/25% glycerol, Photosystem II particles corresponding to 4 μg Chl *a*/ml and, when used, 6 mM hydroxylamine. Excitation after 20 min dark adaption was made at 457 nm (half-band width, 100 nm; intensity, 23 $\mu E \cdot m^{-2} \cdot s^{-1}$). Fluorescence was detected at 680 nm (half-band width, 10 nm).

SDS-polyacrylamide gel electrophoresis was run at constant current using the buffer system of Laemmli [25] with a 12–23% acrylamide gradient and 4 M urea in the gel. For the trypsin experiment, the acrylamide gradient was 10–20% and LiDS replaced SDS.

The 33, 23 and 16 kDa proteins were prepared from spinach thylakoids by ion-exchange chromatography as previously described [26], and rabbit antisera produced by standard immunological methods as in Ref. 27.

Western blotting [28] was performed by electrophoretically transferring the proteins from an SDS-polyacrylamide gel to a nitrocellulose sheet, then incubating the sheet in antibody solution. Antigen-antibody complexes were visualized using horseradish peroxidase-linked goat anti-rabbit IgG, and 4-chloro-1-naphthol, according to Bio-Rad instructions.

Manganese was determined by flameless atomic absorption. The samples were prepared by evaporation, addition of concentrated HNO_3 and H_2O_2 and heating. When approx. 5% of the liquid remained, the samples were made up to 1 ml with distilled water.

Results

Immunological identification of proteins

A striking feature of Photosystem II oxygen-evolving particles from *P. laminosum* was their apparent lack of a polypeptide that might correspond to the 23 kDa polypeptide of higher plants [18,19]. This is confirmed in Fig. 1a, lane 5. No direct evidence can be obtained from the gel for the presence of the Photosystem II 16 kDa polypeptide, because that molecular-weight region is dominated by the phycobiliprotein subunits. The *P. laminosum* particles show a distinct band with nearly identical electrophoretic mobility to the spinach 33 kDa protein (Fig. 1a, lanes 1, 2 and 5). These analyses cannot exclude the possible existence of cyanobacterial proteins corresponding to the spinach 23 and 16 kDa proteins. Apart from the problem of comigration, the identification may be obscured by changes in electrophoretic mobility and staining properties of the cyanobacterial proteins. We therefore used monospecific antibodies against each of the spinach 33, 23 and 16 kDa proteins [27] to see if the *P. laminosum* particles contained cross-reacting polypeptides. Previous studies have shown that antisera against these three proteins show high cross-reactivity with thylakoid preparations from various plants and *Chlamydomonas reinhardtii* (Ryrie, I.J. and Andersson, B., unpublished data). Western blotting of *P. laminosum* particles using the antiserum against the spinach 33 kDa protein showed a polypeptide with cross-reactivity at the same position as the spinach protein (Fig. 1b). In contrast, no polypeptides in the *P. laminosum* Photosystem II particles reacted with sera against either the

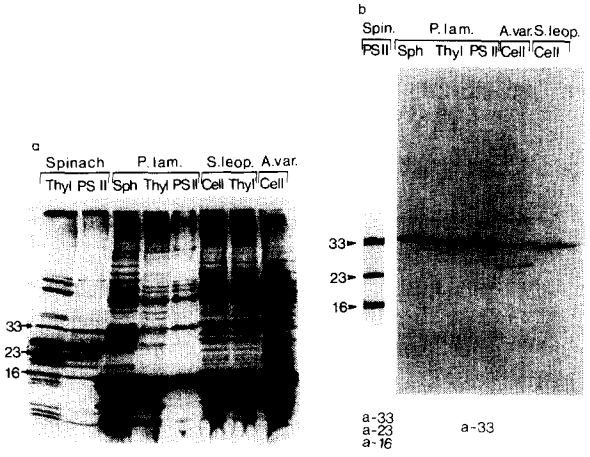


Fig. 1. (a) Polypeptide profiles, as resolved by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining, of spinach thylakoids and Photosystem II particles, and preparations from the cyanobacteria *Phormidium laminosum* (P. lam.), *Synechococcus leopoliensis* (S. leop.), and *Anabaena variabilis* (A. var.). Thyl, thylakoids; PS II, Photosystem II particles; Sph, spheroplasts; cell, cell homogenate. Electrophoresis was run as described by Laemmli [25], employing a 12–23% acrylamide gradient and 4 M urea in the gel. The spinach 33, 23 and 16 kDa proteins are indicated. Chlorophyll loadings per lane were 15 μg for spinach, 5 μg for *P. laminosum* Photosystem II particles and 12.5 μg for all other cyanobacterial samples. (b) Western blotting of an equivalent gel to that in (a), using antisera against the spinach 33 kDa protein. The loading of the cyanobacterial preparations was twice that in (a). Sample abbreviations are as in (a). The western blotting was performed by electrophoretic transfer of the polypeptides from the polyacrylamide gel onto a nitrocellulose sheet. This was incubated in antiserum diluted 3000× for the spinach preparation and 500× for the cyanobacterial preparations. The antibody-labelled polypeptides were visualised by peroxidase-linked secondary antibodies, using 4-chloro-1-naphthol as substrate.

spinach 23 or 16 kDa proteins (not shown).

The negative results from both the Coomassie-stained gels and western blotting give a strong indication that the *P. laminosum* particles do not contain any polypeptides related to the 23 and 16 kDa proteins of higher plants. This could be so, because *P. laminosum* actually lacks these polypeptides or, as has been shown to be the case with mangrove thylakoids [8], the polypeptides may have been lost during the preparation of the membranes or Photosystem II particles. Therefore, thylakoids and sphaeroplasts of *P. laminosum* were subjected to SDS-polyacrylamide gel electrophoresis and western-blotting analyses (Fig. 1 a and b). The thylakoids show no Coomassie-stained band in the 23 kDa region, while the spheroplasts give a complicated polypeptide pattern with numerous polypeptides throughout the whole molecular-weight range. When blotted, both spheroplasts and thylakoids of *P. laminosum* showed a polypeptide cross-reacting with the anti-33 kDa serum (Fig. 1b.) consistent with the observation for the Photosystem II particles. In contrast, the *P. laminosum* spheroplasts did not show any polypeptides crossreacting with the spinach 23 and 16 kDa proteins (Fig. 1b.). Furthermore, the western-blot analyses were carried out for cell homogenates of two other species of cyanobacteria, *Synechococcus leopoliensis* and *Anabaena variabilis*. As can be seen in Fig. 1b, blotting of these species showed the same result as for *P. laminosum*, i.e., cross-reactivity with the spinach 33 kDa protein, but not with the 23 and 16 kDa proteins. In *A. variabilis*, a cross-reacting polypeptide around 25 kDa probably represents a proteolytic fragment of the 33 kDa protein. The same negative results were obtained from three different sera against the 23 kDa and two against the 16 kDa protein. Thus, in cyanobacterial species representative of three of the five taxonomic groups (described by Rippka et al. [29]), there are no proteins showing cross-reactivity with the spinach 23 and 16 kDa polypeptides. In contrast to the 23 and 16 kDa proteins, the 33 kDa protein is present in all the cyanobacterial species and the cross-reactivity with antisera to the spinach protein indicates that this protein has been conserved throughout the course of evolution. Interestingly, we observed strong cross-reactivity between a 7 kDa polypeptide in *P. laminosum* Photosystem II particles and antibodies to spinach cytochrome *b*-559 (unpublished results).

As might be predicted from the immunological results, purified 23 kDa and 16 kDa proteins from spinach were found to have no effect on oxygen evolution when added to *P. laminosum* Photosystem II particles, even at low concentrations of calcium and/or chloride ions (not shown). This was in contrast to the ability of the spinach 23 kDa protein to stimulate oxygen evolution at low Cl^- concentrations in mangrove thylakoids depleted of 23 kDa and 16 kDa proteins. [8].

TABLE I

EFFECT OF GLYCEROL AND SALTS ON OXYGEN-EVOLUTION ACTIVITY OF *P. LAMINOSUM* PHOTOSYSTEM II PARTICLES

O_2 evolution was assayed with 1 mM phenyl-*p*-benzoquinone as acceptor, in 40 mM Mes-NaOH (pH 6.5) or 40 mM Hepes-NaOH (pH 7.5) plus 25% (v/v) glycerol and/or salts as listed below.

pH	Glycerol	O_2 evolution activity (μmol O_2/mg Chl *a* per h)				
		No salt	$CaCl_2$ (10 mM)	$MgCl_2$ (10 mM)	NaCl (25 mM)	$CaAc_2$ (10 mM)
6.5	–	336	830	573	454	447
6.5	+	1265	1917	1956	1602	1363
7.5	–	0	119	79	10	8
7.5	+	1166	1976	1964	1620	1403

Calcium and chloride requirement

In higher plants, the 23 and 16 kDa proteins are thought to be somehow involved in facilitating the binding of the calcium and chloride ions necessary for oxygen evolution [6–10]. How might the absence of these proteins in cyanobacteria influence the requirement for these two ions? Calcium has been shown to be lost more easily from cyanobacterial thylakoids than from higher plants and green algae, indicating a looser binding [30]. However, the lability of the Photosystem II-associated calcium during preparation of thylakoids and Photosystem II particles seems to vary amongst different species of cyanobacteria [20,30–32]. It was suggested previously [19] that the highly active Photosystem II particles from *P. laminosum* do not show a response to addition of calcium because they retain their endogenous calcium. A reexamination of salt effects on these particles (Table I) revealed that marked chloride- and general divalent-cation requirements were evident under all assay conditions. Only in the absence of glycerol, when all activities dropped drastically, could a specific $CaCl_2$ requirement for Photosystem II-catalysed phenyl-*p*-benzoquinone reduction be demonstrated. This calcium effect was only clear at acid pH, because at pH 7.5 the glycerol requirement was almost absolute. The relative stimulation of activity by the various salts listed in Table I exceeds the salt stimulation seen for everted spinach thylakoids retaining the 23 and 16 kDa proteins, but the stimulation is less than seen for everted thylakoids depleted of the two proteins [8]. Possibly other proteins may function in regulating the calcium and chloride binding in cyanobacteria.

Release of proteins from Photosystem II particles of P. laminosum

Treatments known to inhibit oxygen evolution in higher plants by removing one or more of the extrinsic proteins were examined for their effects on the activity and polypeptide pattern of *P.*

TABLE II

EFFECTS OF HIGH CONCENTRATIONS OF SALTS AND ALKALINE TRIS ON ACTIVITY AND MANGANESE CONTENT OF *PHORMIDIUM LAMINOSUM* PHOTOSYSTEM II PARTICLES.

Samples of Photosystem II particles were passed through Sephacryl S-300 columns equilibrated with the buffers shown, then desalted (Sephadex G-25) and concentrated by ultrafiltration. O_2 evolution was assayed in 25% glycerol/10 mM $CaCl_2$/40 mM Mes (pH 6.5)/1 mM phenyl-*p*-benzoquinone. Manganese was assayed by flameless atomic absorption spectrophotometry.

Treatment (additions to 20% glycerol/10 mM $MgCl_2$)	O_2 evolution (μmol O_2/mg Chl *a* per h)	Chl *a*/Mn (mol/mol)
40 mM Mes (pH 6.5)	1851	19.2
1 M $MgCl_2$/40 mM Mes (pH 6.5)	40	22.0
1 M $CaCl_2$/40 mM Mes (pH 6.5)	0	not measured
1 M NaCl/40 mM Mes (pH 6.5)	201	16.5
0.8 M Tris-HCl (pH 8.5)	0	167
Untreated particles	1871	14.0

laminosum Photosystem II particles. Treatment with 0.8 M Tris (pH 8.5) or with 1 M NaCl, $CaCl_2$ or $MgCl_2$ at pH 6.5 strongly inhibited oxygen evolution in *P. laminosum* Photosystem II particles (Table II). Surprisingly, neither Tris (Fig. 2) nor $CaCl_2$ or $MgCl_2$ removed nearly as much of the 33 kDa polypeptide from *P. laminosum* as the treatments do from spinach everted thylakoids. This result was confirmed by the observation that anti-33 kDa antibodies still reacted with the Tris-treated *P. laminosum* Photosystem II particles, but not with Tris-treated spinach Photosystem II preparations (not shown). Note that although the cyanobacterial 33 kDa protein remained largely membrane-bound upon Tris washing, most of the manganese was released (Table II).

Strikingly, only one polypeptide (9 kDa) could be totally removed from Photosystem II particles by 0.8 M Tris, high concentrations of NaCl (Fig. 2), 1 M $CaCl_2$ or $MgCl_2$ (not shown). At the highest NaCl concentrations, small amounts of the

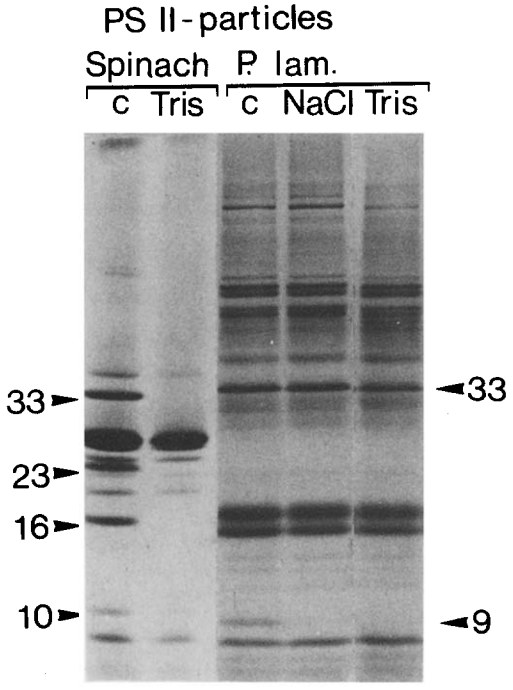
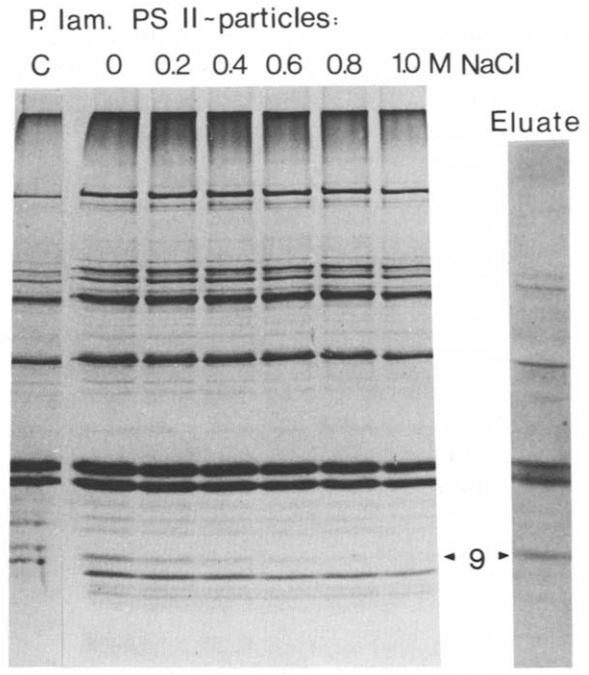


Fig. 2. Effect of treatment with 1 M NaCl (pH 6.5) or 0.8 M Tris-HCl (pH 8.5) on the polypeptide composition of Photosystem II particles from spinach and *Phormidium laminosum* (P. lam.). Particles were treated and electrophoresis was carried out as described in Materials and Methods. c, control; Tris, Tris-washed, NaCl, salt-washed.

phycobiliproteins, the 33 kDa polypeptide and some small polypeptides were also lost. This 9 kDa polypeptide, which can be resolved by electrophoresing at constant current rather than constant voltage, and by inclusion of 4 M urea in the gel, was not resolved by the gel system originally used to characterise the *P. laminosum* particles [18,19]. The 9 kDa protein was not simply proteolysed under high salt conditions, since it could be recovered in a later fraction from the Sephacryl column (Fig. 3a, lane 8 labelled 'eluate'). The protein apparently formed large multimeric aggregates, at least after removal from the Photosystem II particles, since it was retained during subsequent ultrafiltration through a 100 000 kDa cut-off membrane. Loss of Photosystem II activity and the 9 kDa polypeptide from *P. laminosum* Photosystem II particles was not accompanied by significant loss of manganese, except in the case of treatment with 0.8 M Tris, pH 8.5 (Table II). The 9 kDa polypeptide in *P. laminosum* had a higher electrophoretic mobility than the Tris-removable 10 kDa protein from spinach Photosystem II preparations (Fig. 2), and also differed from the higher-plant protein in that the latter is not released by 1 M NaCl or $CaCl_2$ [3].

When the *P. laminosum* Photosystem II particles were treated with increasing concentrations of NaCl (0.2–1 M), the release of the 9 kDa protein (Fig. 3a) correlated quite closely with the deactivation of Photosystem II using either phenyl-*p*-benzoquinone or ferricyanide as acceptor (Fig. 3b). Amounts of the 9 kDa polypeptide showed essentially the same decrease relative to all other polypeptide constituents of the particles, none of which were affected strongly enough by salt to account for the dramatic loss of activity. It has so far not been possible to obtain any restoration of the lost activity by readdition of the fraction containing the released 9 kDa protein or by assaying in the presence of 10–50 mM $CaCl_2$. The salt-treated particles (1 M NaCl) showed a slower fluorescence rise and somewhat lowered F_{max} compared to control particles (Fig. 4). Moreover, addition of the Photosystem II electron donor NH_2OH reversed these effects (not shown). These fluorescence experiments give strong evidence for a role of the 9 kDa polypeptide on the oxidizing side of Photosystem II in *P. laminosum*.



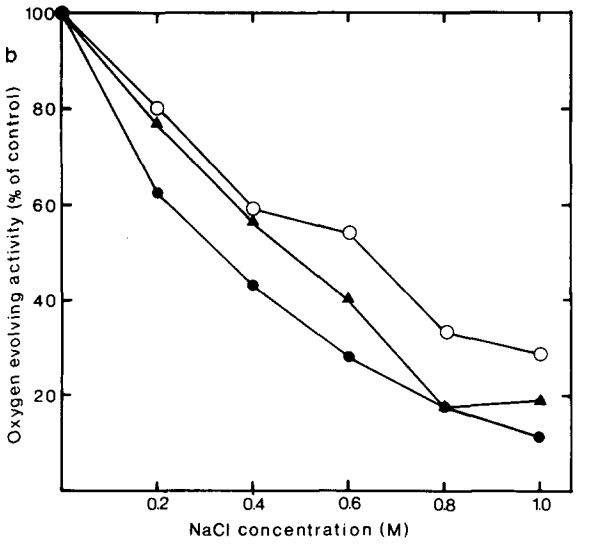


Fig. 3. (a) Effect of treatment with 0–1.0 M NaCl on the polypeptide composition of Photosystem II particles from *Phormidium laminosum*. Samples of Photosystem II particles were passed through Sephacryl S-300 columns, equilibrated with various concentrations of NaCl, then desalted and concentrated. The third fraction eluted from a 1.0 M NaCl-equilibrated column is also shown (eluate). (b). Effect of treatment with 0–1.0 M NaCl on O_2-evolution and the amount of the 9 kDa polypeptide in *Phormidium laminosum* Photosystem II particles, procedure as described in legend to Fig 3a. O_2-evolution was assayed in (filled circles) 25% glycerol, 10 mM $MgCl_2$, 40 mM Hepes (pH 7.5) plus 1 mM phenyl-*p*-benzoquinone (control activity (100%) 1830 μmol O_2/mg Chl *a* per h), or (open circles) 25% glycerol, 10 mM $CaCl_2$, 40 mM Mes (pH 6.0) plus 10 mM ferricyanide (control activity (100%) 1590 μmol O_2/mg Chl *a* per h). The relative protein content in each band was determined by a laser densitometer scanner. The release of the 9 kDa polypeptide (filled triangles) was normalized against two 'non-released polypeptides' at 7 and 45 kDa as well as the total protein content to account for different loadings of gel lanes.

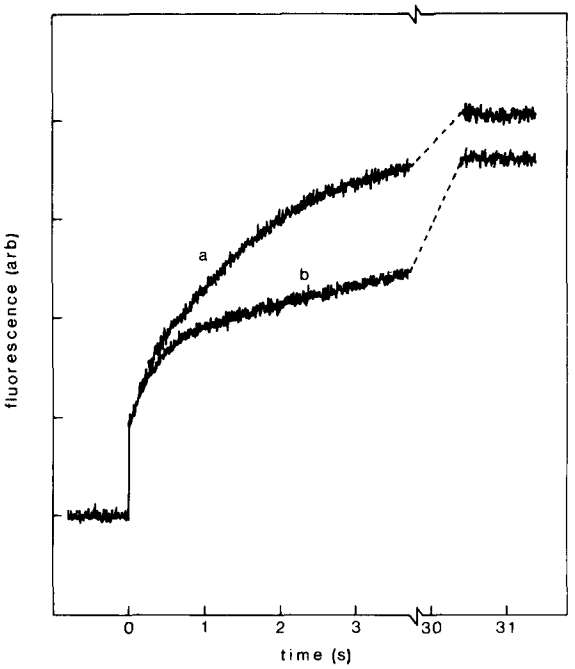


Fig. 4. Fluorescence induction curves for Photosystem II particles of *Phormidium laminosum*. (a) Control; (b) treated with 1 M NaCl.

Trypsin treatment of Photosystem II particles

The Tris and salt washing of *P. laminosum* Photosystem II particles gave no evidence to implicate the 33 kDa protein in the water-oxidation process, since not much of the protein was released. Thus, only its cross-reactivity with the spinach 33 kDa protein would suggest a connection with oxygen evolution. To get more direct evidence we performed trypsin treatment of the particles, a treatment known to affect the 33 kDa protein in everted thylakoids of spinach [33,34]. As shown in Fig. 5a, trypsin digested the 33 kDa polypeptide, but not the 9 kDa polypeptide. Moreover, the trypsin treatment inhibited Photosystem II-catalyzed reduction of both phenyl-*p*-benzoquinone and ferricyanide (Fig. 5b). The latter was inhibited both in the absence and in the presence

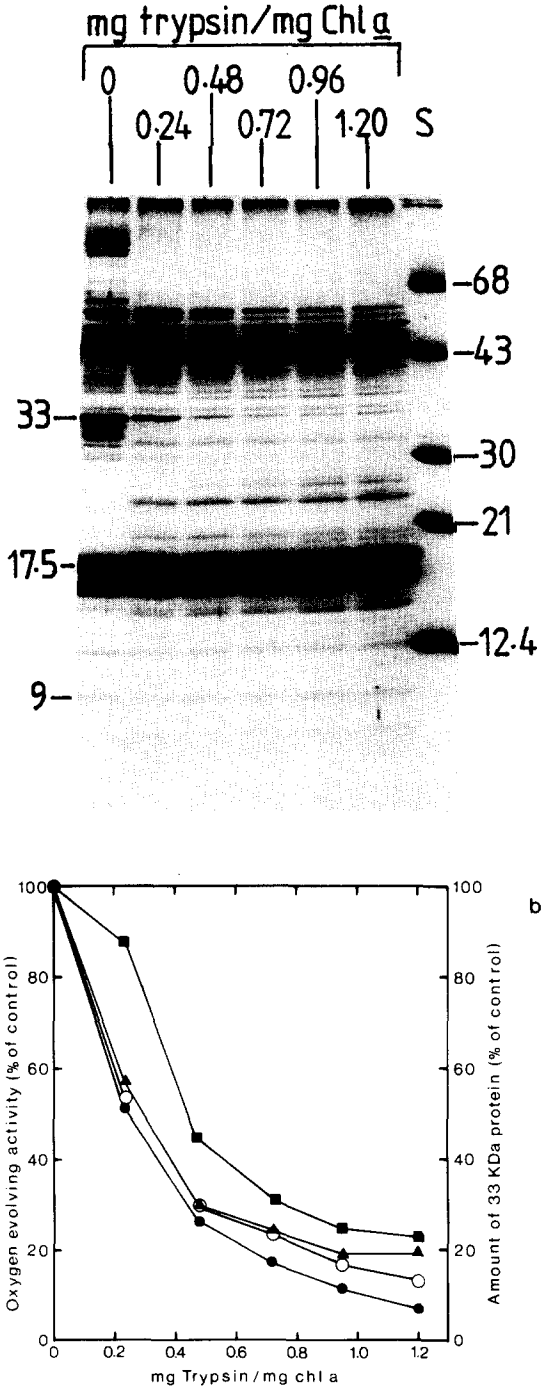


Fig. 5. Effect of trypsin on (a) polypeptide profile and (b) O_2-evolution of *Phormidium laminosum* Photosystem II particles. Samples (20 µl) of Photosystem II particles were incubated at 25°C for 3 min with 20 µl of 50 mM Tricine-NaOH (pH 7.5) containing sufficient trypsin to give the trypsin/Chl *a* ratios shown. O_2-evolution was assayed with (filled circles) 1 mM phenyl-*p*-benzoquinone (control (100%) activity 1420 µmol O_2/mg Chl *a* per h), (open circles) 10 mM ferricyanide (control (100%) activity 1835 µmol O_2/mg Chl *a* per h), or (filled squares) 10 mM ferricyanide + 10 µM DCMU (control (100%) activity 811 µmol O_2/mg Chl *a* per h) in the same buffers as listed in the legend to Fig 3b. Amounts of 33 kDa protein (triangles), calculated from densitometric scans of the gel tracks, were normalised against amounts of 17.5 kDa protein which was not appreciably affected by trypsin.

of DCMU, localizing the major site of inhibition to the oxidizing side of Photosystem II or the reaction centre itself. The course of inhibition corresponded closely with the digestion of the 33 kDa protein, demonstrating that this protein is involved in the oxygen-evolving reaction in cyanobacteria. In some preparations the DCMU-sensitivity of ferricyanide reduction did drop from 50–60% to 25–35% over the series of trypsin treatments, suggesting an additional, but smaller, effect on the herbicide-binding protein.

Discussion

The Photosystem II complex of higher plants includes three closely associated polypeptides, of 33, 23 and 16 kDa, which appear to be of regulatory and structural importance for, but not directly involved in, the catalysis of photosynthetic water oxidation [35]. The present immunological study on the cyanobacterial complex suggests that the 33 kDa protein has been conserved during evolution, but there is considerable evolutionary diversity with respect to the 23 and 16 kDa proteins. Polypeptides of sizes similar to the latter two proteins can be seen in cyanobacterial cells (Fig. 1a). It is therefore possible that these two proteins also belong to cyanobacterial Photosystem II, but are not similar enough to the spinach proteins to give a positive reaction in the immuno-blotting experiment. However, we find this explanation unlikely in light of the observation that no stained band in the 23 kDa region could be found either in oxygen-evolving particles or in thylakoids from *P. laminosum*. Lack of the 23 kDa protein in turn implies lack of the 16 kDa protein, since the latter is both structurally and functionally dependent on the presence of the 23 kDa protein [9,27,36]. We therefore conclude, in view of the considerations made above and the failure of any proteins from three taxonomically diverse strains of cyanobac-

teria to react with antibodies to the plant 23 kDa and 16 kDa proteins, that cyanobacteria do not have these proteins. The procaryotic oxygen-evolving complex appears therefore to be less complicated than the eucaryotic complex. This would be analogous to the cytochrome *c* oxidase complex of respiration, which catalyses the reverse reaction to that of the oxygen-evolving complex [37]. The procaryotic cytochrome *c* oxidase contains only 3 polypeptides, while the eucaryotic complex contains 7–13 polypeptide subunits.

It has been shown that in spinach the 33 and 23 kDa proteins are probably bound to the membrane by intrinsic proteins of 24 and 22 kDa [38]. As indicated above, oxygen-evolving particles of *P. laminosum* show no polypeptides in this molecular weight region. This suggests that the binding of the 33 kDa protein to the intrinsic part of the membrane may differ between cyanobacteria and plants. This is in line with our observation that the cyanobacterial 33 kDa protein was not released to any great extent by treatments like alkaline Tris or high concentrations of $MgCl_2$ or $CaCl_2$, unlike the situation with the corresponding plant protein. The cyanobacterial 33 kDa protein may therefore be located in a more hydrophobic environment. This option may be more open to the cyanobacteria, whose major light-harvesting complex (the phycobilisome) is excluded from the bilayer, than to the higher plants, whose thylakoid membranes must accommodate large amounts of light-harvesting chlorophyll *a/b* protein around Photosystem II.

This study shows a clear requirement for the 33 kDa protein for oxygen evolution in cyanobacteria. In particular, we could show that proteolytic degradation of the protein paralleled loss of oxygen evolution, as has previously been shown in higher plants [34]. We cannot point to any specific function of the 33 kDa protein in cyanobacteria, but in higher plants this protein is supposed to play a stabilizing role for the catalytic manganese [6,7]. One of the most interesting features of our results is the first indications for the requirement of a 9 kDa polypeptide on the donor side of Photosystem II in a cyanobacterium. It is not clear whether the 9 kDa polypeptide in *P. laminosum* corresponds to the recently discovered 10 kDa polypeptide in higher plants [3,38]. If it does, then once again there appear to be some differences in the nature of binding, since it is released by high-salt treatments that leave the spinach polypeptide unaffected [3]. Preliminary results suggest that the spinach 10 kDa polypeptide and the cyanobacterial 9 kDa polypeptide do not cross-react immunologically. Moreover, the *P. laminosum* 9 kDa polypeptide appears not to correspond to an 11 kDa manganese-protein from the cyanobacterium *Plectonema boryanum* [39]. Firstly, the present 9 kDa polypeptide does not contain manganese, and secondly we have found that it is released from thylakoids of both *P. laminosum* and *S. leopoliensis* by brief sonication in the presence of 1 M NaCl (unpublished results), whereas the Mn protein from *Plectonema* appears to be an intrinsic protein that is only released from the thylakoids by detergent. The role for the extrinsic 9 kDa polypeptide in cyanobacterial oxygen evolution remains to be established. It may have a regulatory role, since it is apparently not connected to manganese.

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