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The role of an extrinsic 9 kDa polypeptide in oxygen evolution by Photosystem II particles from *Phormidium laminosum*

Stephen A. Rolfe * and Derek S. Bendall

Department of Biochemistry, University of Cambridge, Cambridge (U.K.)

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Photosystem II particles from the thermophilic cyanobacterium *Phormidium laminosum* have been reported to contain an extrinsic 9 kDa polypeptide that can be dissociated by exposure to a glycerol-free medium, with concomitant loss of activity (Stewart, A.C., Ljungberg, U., Åkerlund, H.E. and Andersson, B. (1985) Biochim. Biophys. Acta 808, 353–362; Stewart, A.C., Siczkowski, M. and Ljungberg, U. (1985) FEBS Lett. 193, 175–179). In the present paper it is shown that a partial recovery of oxygen-evolution activity could be achieved by reconcentrating the particles in the presence of glycerol and an excess of the purified polypeptide. This was shown to be a specific effect of the 9 kDa polypeptide which rebound stoichiometrically. Rapid lowering of the glycerol concentration by dilution gave less inactivation but nearly complete reversibility. When diphenylcarbazide was substituted for water as electron donor no loss of activity was observed. It was concluded that dissociation of the 9 kDa polypeptide caused a reversible inactivation of the oxygen-evolution machinery. Evidence was presented that this could be explained by a slowing, but not complete inhibition, of a dark step in the S-state transitions. No evidence could be obtained for Cl^- or Ca^{2+} concentrating functions of the kind proposed for certain extrinsic polypeptides associated with Photosystem II in chloroplasts.

Introduction

The essential minimum structure of Photosystem II necessary for O_2 evolution is similar in higher plants and cyanobacteria [1], but there seem to be differences in the complement of extrinsic polypeptides, the functions of which are still inadequately defined. With preparations from chloroplasts, polypeptides of approx. 16, 24 and 33 kDa have been intensively studied, and, to a lesser extent, one of 10 kDa (see Refs. 2 and 3 for recent reviews). Cyanobacterial systems have received relatively little attention. Stewart et al. [4] conducted a survey of three species of cyanobacterium, *Phormidium laminosum*, *Synechococcus leopoliensis* (*Anacystis nidulans*) and *Anabaena variabilis*, with antibodies prepared against the chloroplast polypeptides. With all three species, immunoblotting experiments showed cross reaction of a 33 kDa polypeptide with the corresponding spinach antibody, but no cyanobacterial polypeptide cross-reacted with antibodies to the 16 or 24 kDa spinach polypeptides. Similarly, Photosystem II particles from *Synechococcus vulcanus* have been found to contain an extrinsic 34 kDa polypeptide, but nothing corresponding to the 16 or 24 kDa polypeptides [5]. The 34 kDa polypeptide from *S. vulcanus* was capable of partial restoration of O_2-evolution activity of $CaCl_2$-washed spinach Photosystem II particles. Sequence homology of the *wox*A gene from *A. nidulans* R2 with that of spinach suggests [6] that the 33 kDa (so-called manganese-stabilizing) polypeptide is a universal feature of photosynthetic water oxidation.

Attempts to remove extrinsic polypeptides from Photosystem II particles of *P. laminosum* by washing 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 (this is not homologous with the spinach 10 kDa polypeptide), and fluorescence induction curves suggested that the effect was on the donor side [4]. Loss of this polypeptide was also

* Present address: Department of Biology, University of California Los Angeles, Los Angeles, CA, U.S.A.

Abbreviations: DCIP, 2,6-dichlorphenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid; PBQ, phenyl-*p*-benzoquinone; PMSF, phenylmethylsulphonyl fluoride.

Correspondence: D.S. Bendall, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, U.K.

shown to be correlated with loss of O_2-evolving activity on treatment of the particles with a low-glycerol medium [7]. Although these results suggested a role in O_2 evolution, no restoration of activity could be achieved by readdition of the released polypeptide and therefore the possibility could not be ruled out that loss of activity was a secondary irreversible effect.

The aim of the work described in this paper was to demonstrate a role for the 9 kDa polypeptide in the O_2-evolving activity of Photosystem II particles from *P. laminosum* by developing a method for its reversible dissociation.

Materials and Methods

Photosystem II particles were prepared in a laurylmaltoside-containing medium from *P. laminosum* as previously described [8].

The 9 kDa polypeptide was purified by a method based on that of Wallace et al. [9], but which started with a lauryldimethylamine oxide extract of thylakoid membranes rather than Photosystem II particles in order to improve the yield. Thylakoid membrane fragments were incubated for 40 min on ice at a concentration of 1 mg chlorophyll/ml (total 40 mg) in a medium comprising 25% glycerol/20 mM Hepes-NaOH (pH 7.5)/10 mM $MgCl_2$/1 mM PMSF/0.34% lauryldimethylamine oxide and the suspension was centrifuged for 60 min at $110\,000 \times g_{av}$ in a SW50.1 rotor (Beckman L5). To the supernatant were added two vols of a high-salt buffer comprising 4 M NaCl/40 mM Mes-NaOH (pH 6.0)/2 mM PMSF and the mixture was incubated on ice for 15 min before being dialysed against 10 mM sodium phosphate (pH 6.0)/1 mM PMSF. Photosystem II and other membrane proteins were removed by centrifuging for 16 h at $150\,000 \times g_{av}$ in a Beckman 45 Ti rotor, leaving the 9 kDa polypeptide together with some phycobilins and cytochrome *c*-549 in the supernatant. This was used as the source of crude 9 kDa polypeptide. Further purification was achieved by passing the crude material through a column of CM-Sepharose CL-6B (2.5 cm diameter, 80 ml bed volume) equilibrated with 10 mM sodium phosphate (pH 6.0)/1 mM PMSF. Most of the phycobiliproteins passed straight through the column leaving the 9 kDa polypeptide, cytochrome *c*-549 and allophycocyanin bound. The unbound fraction contained some protein with a molecular mass of about 9 kDa, but this had a lower p*I* than the corresponding bound fraction, as determined by two-dimensional polyacrylamide gel electrophoresis. Bound proteins were eluted with a gradient of NaCl (0–0.3 M) in 10 mM phosphate/1 mM PMSF. The 9 kDa polypeptide was eluted at about 150 mM NaCl behind a pale blue fraction containing allophycocyanin and ahead of a bright red band of cytochrome *c*-549. Fractions containing the 9 kDa polypeptide were pooled, dialysed against 10 mM phosphate (pH 6.0)/1 mM PMSF and concentrated with Aquacide (Calbiochem). This preparation was used as the source of purified polypeptide.

Polypeptides were analysed by electrophoresis on 12–22½% gradient polyacrylamide gels containing 4 M urea and lithium dodecylsulphate as described in Ref. 4, except that 18% gels were used during the purification of the 9 kDa polypeptide. The apparent molecular mass of the 9 kDa polypeptide was actually 8.2 kDa (CNBr fragment of myoglobin as marker), but its true molecular mass is 11 263 Da by calculation from the nucleotide sequence of the gene [9]. We refer to it as the 9 kDa polypeptide in conformity with previous publications.

Oxygen-evolution activity was measured at 25 °C with a Hansatech oxygen electrode. Samples were suspended in 10 mM $MgCl_2$/25% glycerol containing either 40 mM Mes-NaOH (pH 6.0) or 20 mM Hepes-NaOH (pH 7.5) unless otherwise specified, and were illuminated with saturating red light (Schott RG 610 filter) after addition of 10 mM potassium ferricyanide or 1 mM PBQ as electron acceptor. Note that 25% glycerol was present in the medium for all assays.

The reduction of DCIP was followed by measuring the decrease in absorbance at 518 nm in a computer-controlled single-beam spectrophotometer (Applied Photophysics, London). Actinic light from a 100 W tungsten-halogen lamp was passed through a red filter (Schott RG 630) and transmitted to the sample by a bifurcated fibre light-guide. The photomultiplier was shielded by a 1 cm layer of saturated copper sulphate and a 580 nm short-pass interference filter (Oriel 70317). The sample was added to 1 ml of buffer comprising 20 mM Mes (pH 6.0)/10 mM $MgCl_2$/25% glycerol/70 μM DCIP. When required, diphenylcarbazide was added to the cuvette (final concentration 0.5 mM) from a 50 mM solution in ethanol which was freshly prepared every 2 h and kept in darkness at 0 °C. The non-enzymic rate of reduction of DCIP by diphenylcarbazide was subtracted from the results. Measurements were made at 518 nm to avoid interference from bleaching of sample pigments. An absorption coefficient of 7770 $M^{-1} \cdot cm^{-1}$ for DCIP at 518 nm was measured with the assumption of an absorption coefficient of the basic form of 22 000 $M^{-1} \cdot cm^{-1}$ at 600 nm [10].

Fluorescence induction curves were measured in the single-beam spectrophotometer with no measuring light. Actinic light was filtered through a broad-band blue interference filter (Oriel 5754) and a 19.1% neutral density filter and activated by a computer controlled shutter with an opening time of 3 ms. The photomultiplier was shielded with a red glass filter (Schott RG630) and a 685 nm narrow-band interference filter (Balzers B40). The signal was sampled and stored in a Datalab DL902 transient recorder before transfer to the computer. The sample contained 1.2 μg chlorophyll in 1 ml of 40 mM Mes (pH 6.0)/10 mM $MgCl_2$/25% glycerol.

If required, 10 μM DCMU and 6 mM hydroxylamine were added just prior to the onset of illumination.

Chlorophyll *a* concentrations were determined by the method of Arnon et al. [11].

Results

Reversible inhibition of oxygen evolution by dilution into low-glycerol buffer

The difficulty in obtaining a reversible dissociation of the 9 kDa polypeptide reported in Refs. 4 and 7 was likely to be due to the time required for separation and reconstitution. The loss of activity following dilution of Photosystem II particles into a low-glycerol buffer was therefore investigated, since this procedure was rapid and probably represented the mildest method by which dissociation of the polypeptide could be achieved.

Time-courses for loss of oxygen evolution on 10-fold dilution of Photosystem II particles into a buffer containing no glycerol are shown in Fig. 1. A relatively stable activity, which was about 50% of the original, was achieved after 40 min incubation at 4°C, whereas no loss of activity occurred over this period when the particles were diluted into buffer containing 25% glycerol. The same underlying process was probably occurring whether the electron acceptor was ferricyanide or PBQ, because similar progress curves were observed, except that with PBQ (data not shown) the initial slight stimulation was not seen.

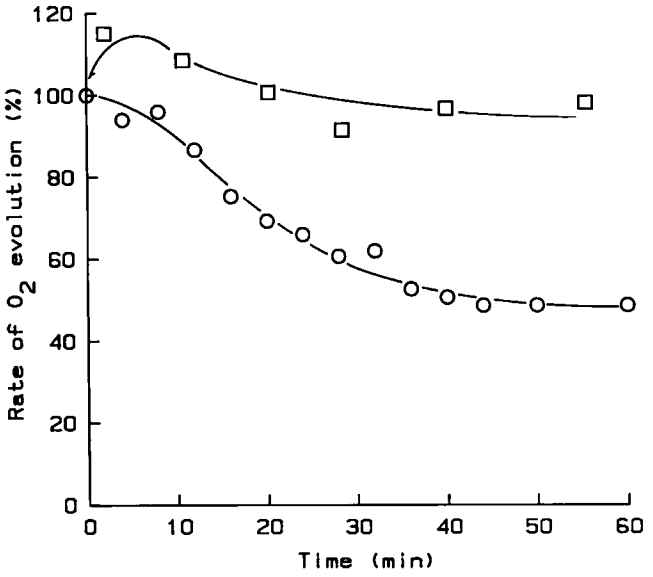

Fig. 1. Inactivation of oxygen evolution by dilution of Photosystem II particles into a glycerol-free medium. Photosystem II particles (315 μg chlorophyll/ml) were diluted 10-fold into either 40 mM Mes (pH 6.5)/10 mM $MgCl_2$ (circles) or 40 mM Mes (pH 6.5)/10 mM $MgCl_2$/25% glycerol (squares) and incubated at 0°C. Samples were withdrawn at the times shown and assayed immediately in pH 6.0 buffer with ferricyanide as electron acceptor. The zero-time rate was 2550 μmol O_2/mg chlorophyll per h.

After incubation in the low-glycerol medium for 40 min, activity could be recovered by readdition of glycerol to 25% followed by concentration of the particles by centrifugation in a Centricon-30 microconcentrator for 60 min at 4300 × *g*. The activity was restored from an average value for the diluted sample of 55% of the original to an average value of 100% with ferricyanide and 69% with PBQ as acceptors. Control experiments in which the glycerol concentration was maintained at 25% throughout showed no change in activity. Measurements of rates of DCIP reduction showed that dilution lowered the activity to 42% of the control (the control rate was 320 μmol DCIP/mg chlorophyll per h), and reconcentration with glycerol restored it to 96% when water was the electron donor. On the other hand, when the assay medium contained diphenylcarbazide, which can act as an artificial donor to Photosystem II, no inhibition was observed. All rates of DCIP reduction were strongly inhibited by 10 μM DCMU.

These results showed that dilution and reconcentration caused a reversible loss of activity on the donor side of Photosystem II which could be tentatively ascribed to reversible dissociation of the 9 kDa polypeptide. The properties of particles that had been incubated for 40 min in a low-glycerol medium, as above, were investigated further in order to characterize the nature of the inhibition.

Properties of low-glycerol-treated particles

Activity can frequently be restored to Photosystem II preparations from chloroplasts depleted of extrinsic polypeptides by addition of a high concentration of Cl^- or Ca^{2+} [1,2]. Although Stewart et al. [4,7] were unable to reproduce such effects with *Phormidium* preparations, secondary structural changes might have prevented them. Table I shows the results of experiments on the effects of various salts on activities when the particles were diluted into a low glycerol medium under conditions that allowed the inhibition to be reversed. Although higher rates were observed in the diluted samples in the presence of $MgCl_2$ or $CaCl_2$ than in their absence, as was expected from the known properties of *Phormidium* Photosystem II particles [12], the stimulations were less than with the undiluted samples, i.e., the salts were unable to reverse the effect of low glycerol treatment. A fairly low concentration of NaCl (20 mM) inhibited the rates with the low-glycerol samples but not with those kept in 25% glycerol.

Fluorescence induction curves were measured with fresh samples, after low-glycerol treatment for 40 min on ice, and after reconcentration of the particles in the presence of 25% glycerol. Rates of O_2 evolution at each stage and details of the procedure are given in Table II. Note that the suspension required dark equilibration for 25 min at room temperature to allow complete reoxidation of the acceptor pool. This was done at the begin-

TABLE I

The effects of salts on oxygen evolution by low-glycerol-treated Photosystem II particles

Photosystem II particles were diluted 10-fold into 40 mM Mes/10 mM $MgCl_2$ (pH 6.5) with or without 25% glycerol and incubated on ice for 40 min. Oxygen evolution was then assayed in 40 mM Mes/25% glycerol (pH 6.0) with additions as shown below. Either 10 mM potassium ferricyanide or 1 mM PBQ was added as electron acceptor. All rates are averages of duplicate measurements and expressed as μmol O_2/mg chlorophyll per h.

Addition	Ferricyanide			PBQ		
	Rate		relative rate (%)	Rate		relative rate (%)
	control	low glycerol		control	low glycerol	
None	1849	967	52.3	2387	1735	72.3
10 mM $MgCl_2$	2663	1086	40.8	2696	1838	68.2
10 mM $CaCl_2$	3002	1103	36.7	3022	1941	64.2
30 mM $CaCl_2$	3223	1255	38.9	2576	1597	62.0
20 mM NaCl	2205	560	25.4	2731	1511	55.3
10 mM $MgCl_2$/ 40 mM NaCl	2273	729	32.1	2336	1110	47.5

ning of the experiment and again after restoration of the normal glycerol concentration, because of the difficulty of reconcentration in absolute darkness. Nevertheless, substantial recovery of activity was observed with ferricyanide as acceptor, and a small but significant recovery with PBQ. In contrast to the result obtained by Stewart et al. [4], no significant difference was observed between the induction curves of the fresh particles and after dilution into low-glycerol buffer (Fig. 2). Reconcentration with 25% glycerol led to a small drop in the value of F_{max} (and F_v) which was associated with a drop in the acceptor pool size from about 2.8 equivalents per reaction centre to 2.2 (Table II). This was consistent with the irreversible loss of activity observed with PBQ as acceptor (Table II) and suggests that electron transfer from Q_A to Q_B was impaired in one-third of the centres. The results also show that the pools associated with each reaction centre contain about one molecule of plastoquinone each and do not interact with each other. This is consistent with the previous conclusion from the exponential rise of fluorescence that the particles are essentially monomeric [13]. The addition of hydroxylamine immediately before measurement of induction kinetics increased F_v, indicating that the donor side of some centres had been activated, but the effect was no greater after dissociation of the 9 kDa polypeptide by low-glycerol treatment (Table II).

There were two possible explanations for the failure to observe an effect of low-glycerol treatment on the fluorescence induction kinetics that would be consistent with the conclusion of the previous section that the effect was on the donor side of Photosystem II. The

TABLE II

Fluorescence characteristics and oxygen-evolution activity of low-glycerol-treated and reconcentrated Photosystem II particles

Photosystem II particles were thawed, tested for activity ('Untreated') and dark adapted for 25 min at room temperature. Samples were withdrawn for measurement of fluorescence induction curves and O_2-evolution activity ('Dark adapted'). The dark-adapted particles were diluted 10-fold into 40 mM Mes/10 mM $MgCl_2$ (pH 6.5) and kept on ice for 40 min. Samples were withdrawn as before ('Diluted'). After addition of glycerol (25%), the suspension was reconcentrated 10-fold by centrifugation in a Centricon-30 and dark adapted again for 25 min at room temperature. Samples were again withdrawn for measurement ('Reconcentrated'). Rates of O_2 evolution were measured at pH 6.0 with both acceptors. See Materials and Methods for further details.

Addition	Fluorescence (arbitrary units)		Acceptor pool (equivalents/ reaction centre)	Oxygen evolution (μmol O_2/mg Chl per h)	
	F_{max}	F_0		ferricyanide	PBQ
Untreated				3240	2600
Dark adapted					
None	183	39	2.8	2780	2290
DCMU	169	50			
NH_2OH	230	41	3.1		
NH_2OH/DCMU	212	55			
Diluted					
None	181	37	3.0	1730	1390
DCMU	163	44			
NH_2OH	226	38	3.2		
NH_2OH/DCMU	207	48			
Reconcentrated					
None	167	42	2.2	2470	1580
DCMU	162	45			
NH_2OH	219	45	2.2		
NH_2OH/DCMU	202	49			

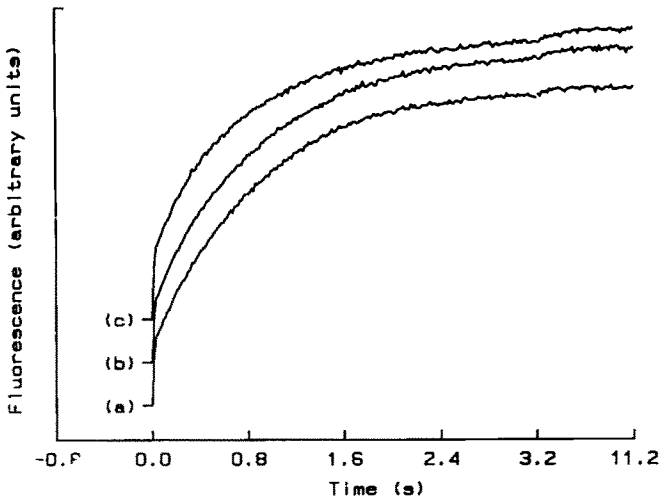

Fig. 2. Fluorescence induction curves of low-glycerol-treated Photosystem II particles. The traces shown were taken on the samples described in Table II in the absence of DCMU and hydroxylamine. (a) untreated sample; (b) after incubation in low-glycerol medium; (c) after reconcentration in the presence of 25% glycerol.

first was that the effect was on the O_2 evolution step itself, which would not have occurred during fluorescence induction because of the small size of the total acceptor pool. The second was that the inhibition of steady-state O_2 evolution was the result of the slowing of a dark step in the S-state transitions rather than the complete inhibition of the O_2 evolution reaction. In the latter case no effect would have been observed on the fluorescence measurements because of the light-limiting conditions.

With spinach Photosystem II particles the loss of O_2-evolution activity caused by dissociation of the 33 kDa extrinsic polypeptide was shown to disappear at low light intensity, and the effect was explained by the slowing of the S_3 to S_0 transition [14]. Fig. 3A shows the effect of light intensity on the steady-state rate of O_2 evolution in the presence of ferricyanide as acceptor with normal and low-glycerol-treated *Phormidium* particles. The inhibition was about 45% at high intensity and became zero at the lowest intensity used. This result demonstrated that dissociation of the 9 kDa polypeptide slowed a dark step in the S-state transitions and would be most readily explained by a decrease in the rate of the O_2 evolution step, because this is normally the slowest of the S-state transitions, but an effect on a lower S-state transition cannot be excluded. However, if the sample were homogeneous, one would expect the inhibition to increase continuously with light intensity, but this was not the case, as is shown in Fig. 3B, where the rate is plotted as a function of the average turnover time per electron in the control sample. At longer turnover times the rate as a percentage of the uninhibited increased linearly, as would be expected, but below 40 ms the inhibition remained constant. From this we conclude that the low-glycerol-treated particles were heterogeneous, about 55% behaving normally, and the remainder exhibiting a slowed S-state transition with a turnover time of about 110 ms. This is consistent

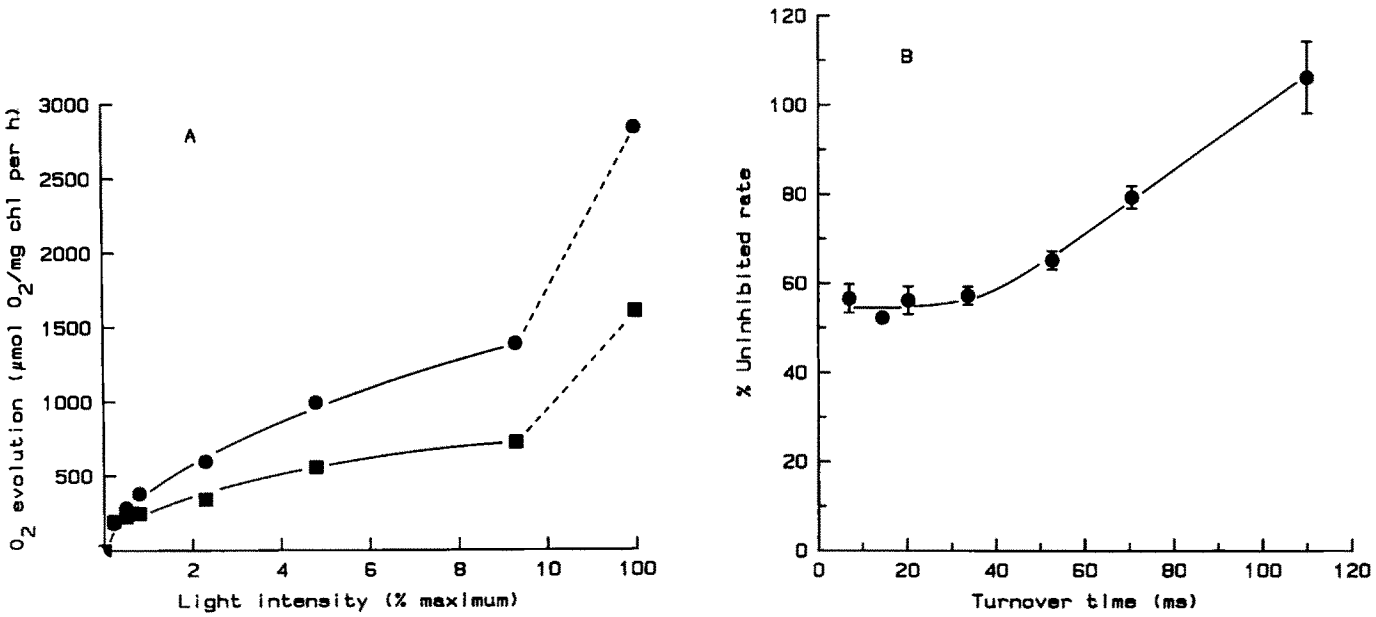

Fig. 3. Effect of light intensity on oxygen-evolution activity of low-glycerol-treated Photosystem II particles. The particles (202 µg chlorophyll/ml) were diluted 10-fold into 40 mM Mes (pH 6.5)/10 mM $MgCl_2$ with or without 25% glycerol and incubated at 0 °C for 40 min. Samples were withdrawn for assay at pH 6.0 with ferricyanide as electron acceptor. (A) rate of O_2 evolution with (circles) and without (squares) glycerol vs. light intensity expressed as a percentage of the maximum (53 mW/cm^2); (B) activity of the low-glycerol sample as a percentage of the control sample vs. the turnover time of the control sample calculated, assuming that all centres were active and contained 50 molecules of chlorophyll *a*. Mean of three determinations (± S.D.).

with the finding (Ref. 7 and see below) that low-glycerol treatment did not completely dissociate the 9 kDa polypeptide; it has little or no effect on the 33 kDa polypeptide [7] or on the Mn content [12].

Restoration of oxygen evolution by the 9 kDa polypeptide

In the above experiments the inhibition of O_2 evolution by dilution into a low-glycerol medium was reversed by reconcentrating the particles in the presence of 25% glycerol. We next sought to show that this was due specifically to rebinding of the 9 kDa polypeptide.

When Photosystem II particles were inhibited by 10-fold dilution into a glycerol-free buffer, partial reactivation occurred by addition of 25% glycerol, without reconcentration (Table III). When a large excess of crude 9 kDa polypeptide was added together with the glycerol, the activity was completely restored. It was notable that this was true with both PBQ and ferricyanide as acceptors.

Reactivation by purified 9 kDa polypeptide and other protein fractions was studied with a preparation of Photosystem II particles, from which as much as possible of the endogenous 9 kDa polypeptide had been removed by passage through a column of Sephacryl S-300 equilibrated with a glycerol-free buffer. The activities of column-treated and reconstituted samples were measured with both ferricyanide and PBQ as acceptors, and the quantity of total 9 kDa polypeptide in the suspension (bound and free) was determined by polyacrylamide gel electrophoresis and densitometry of the Coomassie stained gel in which the size of the 6 kDa cytochrome *b*-559 peak was used as an internal standard (see Fig. 4). The results of these experiments are shown in Table IV. Passage of Photosystem II particles through the column resulted in a loss of O_2-evolution activity and depletion of the 9 kDa polypeptide. Reconcentration with either crude or purified 9 kDa polypeptide in the presence of glycerol led to significant, although partial, restoration of ferricyanide-supported activity, and a smaller restoration of activity with PBQ, but reconcentration with cytochrome *c*-549 gave no recovery. The initial eluant from the CM-Sepharose column used to purify the 9 kDa polypeptide contained a second 9 kDa polypeptide of relatively low p*I*, but this was also shown to be inactive (Expt. 2). A large excess of the 9 kDa polypeptide was required to achieve maximal restoration, as shown by the smaller reactivation in Expt. 2 (6-fold excess) than in Expt. 1 (10-fold excess). This was probably due to the fact that the protein aggregated in solution at pH 6.5. When particles which had been reconstituted with crude 9 kDa polypeptide were separated from unbound protein by passage through a second Sephacryl S-300 column equilibrated with 25% glycerol/10 mM $MgCl_2$/40 mM Mes (pH 6.5), they showed a 9 kDa/cytochrome *b*-559 ratio of 0.87, which was very similar to that of the untreated sample. Fig. 4 shows that passage through the first Sephacryl column also removed a 12 kDa polypeptide. Although this has not been identified, it is present to a variable extent in Photosystem II preparations and is probably a contaminant [8]. These experiments showed that recovery of activity was a specific effect of the 9 kDa polypeptide.

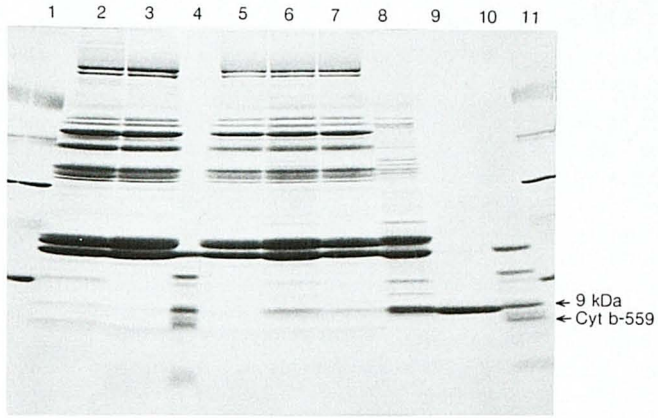

Fig. 4. LDS-polyacrylamide gel of depleted and reconstituted Photosystem II particles. The samples used were those of Expt. 1 in Table IV. Lanes 1 and 11, high-molecular-mass standards (bovine serum albumin, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 30 kDa, soyabean trypsin inhibitor, 20.5 kDa and cytochrome *c*, 12.2 kDa); lane 2, untreated particles (3.5 μg chlorophyll); lane 3, column-treated particles (2.5 μg chlorophyll); lanes 4 and 10, low-molecular-mass markers (myoglobin, 17 kDa, and CNBr fragments of myoglobin, 14.4, 8.2, 6.2 and 2.5 kDa); lanes 5–7, depleted particles (2 μg chlorophyll) reconstituted with no protein (lane 5), crude 9 kDa polypeptide (lane 6), or pure 9 kDa polypeptide (lane 7); lane 8, crude 9 kDa polypeptide; lane 9, pure 9 kDa polypeptide.

TABLE III

Restoration of oxygen evolution by addition of glycerol and crude 9 kDa polypeptide to low-glycerol-treated Photosystem II particles

Photosystem II particles were thawed, tested for activity ('Untreated'), diluted 10-fold into 40 mM Mes (pH 6.5)/10 mM $MgCl_2$ and incubated for 30 min on ice. Samples were withdrawn immediately for assay with the additions given below; an excess of the crude 9 kDa polypeptide was used. Assays with ferricyanide as acceptor were at pH 6.0 and with PBQ at pH 7.5.

Sample	Additions	Oxygen evolution (μmol O_2/mg Chl per h)	
		ferricyanide	PBQ
Untreated		1970 (100%)	4200 (100%)
Diluted	None	1080 (55%)	2270 (54%)
	25% glycerol	1420 (72%)	3430 (82%)
	25% glycerol/ 9 kDa protein	1940 (98%)	4030 (96%)

Discussion

The experiments described above demonstrate conclusively that the reversible dissociation of the extrinsic

TABLE IV

Reconstitution of depleted Photosystem II particles with purified 9 kDa polypeptide

Photosystem II particles were thawed and samples taken for activity measurements and polypeptide analysis. The suspension was then passed through Sephacryl S-300 as described in Ref. 7. Analyses were made on the chlorophyll-containing fraction from the column, untreated and after reconcentration in a Centricon-30 microconcentrator in the presence of 25% glycerol and an excess of the protein fraction given below. Activities with ferricyanide were measured at pH 6.0 and with PBQ at pH 7.5. The concentration of 9 kDa polypeptide in the suspension at each stage was measured relative to the 6 kDa band of cytochrome *b*-559 by polyacrylamide gel electrophoresis and densitometry of the Coomassie stained gel. Low p*I* 9 kDa protein, unbound fraction from CM-Sepharose column (see Materials and Methods).

Sample	Oxygen evolution (μmol O_2/mg Chl per h)		9 kDa protein (rel. concn.)
	ferricyanide	PBQ	
Expt. 1			
Untreated	1830 (100%)	3930 (100%)	0.95
Column treated	770 (42%)	1020 (26%)	0.37
Reconcentrated with:			
no addition	740 (41%)	980 (25%)	–
crude 9 kDa protein	1220 (67%)	1390 (35%)	10
pure 9 kDa protein	1310 (71%)	1290 (33%)	10
Expt. 2			
Untreated	3410 (100%)	2640 (100%)	0.82
Column treated	910 (27%)	670 (25%)	0.37
Reconcentrated with:			
no addition	1000 (29%)	850 (32%)	0.26
crude 9 kDa protein	1860 (55%)	1200 (45%)	9.94
pure 9 kDa protein	1480 (43%)	990 (42%)	5.52
low p*I* 9 kDa protein	1010 (29%)	770 (29%)	5.95
cytochrome *c*-549	1110 (32%)	880 (33%)	0.32

9 kDa polypeptide from *Phormidium* Photosystem II particles is associated with a reversible loss of O_2-evolution activity. In experiments designed to show that the restoration of activity was a specific effect of the 9 kDa polypeptide, the recovery was only partial, but this could probably be explained by a more general lability of the system in the absence of glycerol and the long time necessary for manipulation of the samples. The most rapid and effective procedure was to cause dissociation by incubation in a low-glycerol medium at 0 °C for 30–40 min and rebinding by reconcentration in the presence of 25% glycerol and excess crude 9 kDa polypeptide. The loss of activity with both ferricyanide and PBQ as acceptor was fully reversible, and it was reasonable to assume that under these conditions, too, the change of activity was a specific effect of the 9 kDa polypeptide.

The main conclusion is that we have confirmed the suggestion of Stewart et al. [4] that the presence of the 9 kDa polypeptide in a bound state is necessary for normal photosynthetic oxygen evolution in this cyanobacterium. The evidence strongly favours an effect on normal S-state advancement, probably a dark step involved in the S_3 to S_0 transition, but further investigation by more direct methods is required. We have also confirmed, under reversible conditions, that the role of the polypeptide cannot be substituted by specific salts, in the manner that is known for extrinsic polypeptides associated with higher-plant Photosystem II. These experiments also suggest that the Cl^- and Ca^{2+} concentrating functions of the 16 and 24 kDa polypeptides, which are present in chloroplasts but absent from *P. laminosum* and some other cyanobacteria, are not an essential feature of photosynthetic oxygen evolution.

The 9 kDa polypeptide has not been found in Photosystem II preparations from chloroplasts and its distribution in cyanobacteria is not yet known. One possibility is that it is a feature of the thermophilic nature of *P. laminosum* and helps to stabilize the system. Another possibility is that it forms part of a control system, involving an unknown effector, that turns off oxygen evolution and allows dissipative cycling around Photosystem II without interfering in Photosystem I function. However, an answer to the question of the function of the polypeptide will depend on further experiments.

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