Glycerol stabilizes oxygen evolution and maintains binding of a 9 kDa polypeptide in photosystem II particles from the cyanobacterium, *Phormidium laminosum*

Alison C. Stewart, Martin Siczkowski and Ulf Ljungberg*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, England and **Department of Biochemistry, University of Lund, Box 124, S-221 00 Lund, Sweden*

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High concentrations of glycerol ($\geq 20\%$ v/v) stabilize oxygen evolution in photosystem (PS) II particles from the thermophilic cyanobacterium, *Phormidium laminosum*. Treating PS II particles with lower glycerol concentrations inhibits activity and, in parallel, detaches a 9 kDa polypeptide from PS II. These results suggest that the 9 kDa polypeptide is essential for PS II activity, and that it is bound to PS II primarily by hydrophobic interactions that are strengthened in vitro by glycerol. The 9 kDa polypeptide is distinct from cytochrome *b*-559 and does not appear to be associated with manganese or with the calcium or chloride requirements for oxygen evolution.

Oxygen evolution *Photosystem II* *Thermophilic cyanobacterium* *9 kDa polypeptide* *Glycerol requirement* *Hydrophobic interaction*

1. INTRODUCTION

The composition of the oxygen-evolving complex in the prokaryotic cyanobacteria has become the focus of increasing interest. A recent study using immunoblotting [1] has shown that, of the 33, 23 and 16 kDa proteins that have been shown to be associated with oxygen evolution in the higher plants and algae (review [2]), only the 33 kDa polypeptide has a clear counterpart in the cyanobacteria. Moreover, evidence was presented [1] for the association of a 9 kDa polypeptide with the photosynthetic oxygen-evolving complex in highly active PS II particles from the cyanobacterium, *Phormidium laminosum*. This polypeptide could be removed from the particles by treatment with 0.8 M alkaline Tris or 1 M salts ($NaCl$, $MgCl_2$, $CaCl_2$), with concomitant loss of oxygen evolution.

The cyanobacterial oxygen-evolving complex appears not only to be different structurally from the complex of eukaryotic organisms, but to display some different properties in vitro. Unlike higher-plant thylakoids, most cyanobacterial preparations are dependent, for maximal oxygen-evolution activity, on the presence of high concentrations of substances such as sucrose (1 M) or glycerol (20–25%, v/v) in the storage and assay media. This striking requirement was first reported many years ago [3,4] and has been reiterated continually in subsequent reports (e.g. [5–8]) but to date no satisfactory explanation has been advanced, and the glycerol/sucrose requirement for oxygen evolution in cyanobacterial preparations has remained a puzzling empirical observation.

Here, we are able to show a clear correlation between the glycerol requirement for oxygen evolution in PS II particles from *P. laminosum* and the binding of a 9 kDa polypeptide to PS II.

Abbreviations: Mes, 4-morpholineethanesulfonic acid; chl, chlorophyll; LiDS-PAGE, lithium dodecyl sulfate polyacrylamide gel electrophoresis; PS, photosystem

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2. MATERIALS AND METHODS

PS II particles were prepared from *P. laminosum* as in [9], except that the concentration of dodecyldimethylamine oxide used to extract PS II from the thylakoids was 0.35%. The PS II particles were concentrated to approx. 0.1 mg chl/ml in an Amicon ultrafiltration cell (YM100 membrane). A chromatographic method was used for treating the particles with different concentrations of glycerol. 4-ml samples of PS II particles were passed through 2.5 × 10 cm columns of Sephacryl S-300 (bed volume 50 ml) equilibrated with 10 mM $MgCl_2$, 40 mM Mes, pH 6.5, and a concentration of glycerol between 0 and 25% (v/v). Twelve 2-ml fractions were collected and pooled in 3 groups: fractions 1–4 (elution volume 29 ml) were dark green, fractions 5–7 (elution volume 36 ml) paler bluish green, and fractions 8–12 (elution volume 44 ml) pale blue. Each group of fractions was immediately restored to 25% in glycerol by an addition from a stock of 75% glycerol in 10 mM $MgCl_2$, 40 mM Mes, pH 6.5, then concentrated to approx. 1.5 ml by ultrafiltration through an Amicon YM100 membrane (fractions 1–4 and 5–7) or YM10 membrane (fractions 8–12). (We checked that the same result was obtained when fractions 1–4 were filtered through a YM10 membrane, however a YM100 membrane was normally used for greater rapidity.) Fractions 1–4 (containing 80% of the chlorophyll) were used for activity measurements and LiDS-urea-PAGE. Fractions 5–7 (essentially the tail of the first peak) were not analysed further. Fractions 8–12, containing proteins released and separated from the PS II particles during the chromatographic treatment, were kept for examination by LiDS-urea-PAGE.

Chl *a* was assayed as in [10]. Oxygen evolution was measured at 25°C using an oxygen electrode [9]. The assay medium contained 25% glycerol, 40 mM Mes, pH 6.0, 10 mM ferricyanide, PS II particles equivalent to 2–3 μg chl *a*/ml and either 10 mM $CaCl_2$ or other salts as detailed in table 1. LiDS-urea-PAGE was performed as in [1].

3. RESULTS

When PS II particles were subjected to Sephacryl S-300 chromatography in buffers containing either 0% or 25% glycerol, the activity of particles eluted from a 0% glycerol-equilibrated column was found to be only 595 μmol O_2/mg chl *a* per h, vs 1982 μmol O_2/mg chl *a* per h for PS II particles maintained at 25% glycerol. Polypeptide profiles revealed only one difference that appeared to be substantial enough to account for this drop in activity: the loss of a polypeptide of 9 kDa from PS II particles chromatographed at 0% glycerol (fig.1). An advantage of using the gel-filtration technique for treating the PS II particles was that the later (i.e. lower M_r) fractions from the columns could be examined directly for polypeptides removed by the treatment. Fractions 8–12 from both 0 and 25% glycerol-equilibrated columns

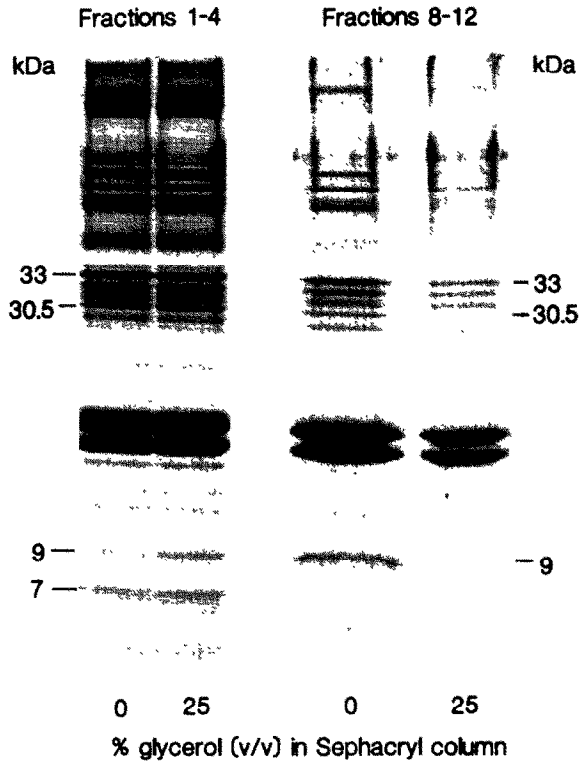

Fig.1. Polypeptide profiles of *P. laminosum* PS II particles chromatographed on Sephacryl S-300 in the presence of 0% or 25% (v/v) glycerol. Chromatography, collection of fractions and electrophoresis were carried out as described in section 2. Lane loadings: fractions 1–4, PS II particles corresponding to 2 μg chl *a*; fractions 8–12, a sample corresponding to 3% of the total volume of these fractions.

contained phycobiliproteins and small amounts of polypeptides in the 30–35 and 50–60 kDa regions, but only the fractions from the 0% glycerol column contained the 9 kDa polypeptide (fig.1).

To quantitate the relationship between the 9 kDa polypeptide, glycerol concentration and activity, PS II particles were treated with a series of buffers containing decreasing concentrations of glycerol, and the activity and polypeptide levels of the treated PS II particles were monitored. As fig.2 shows, the level of the 9 kDa polypeptide dropped in parallel with activity. In contrast, smaller changes in the levels of phycobiliproteins

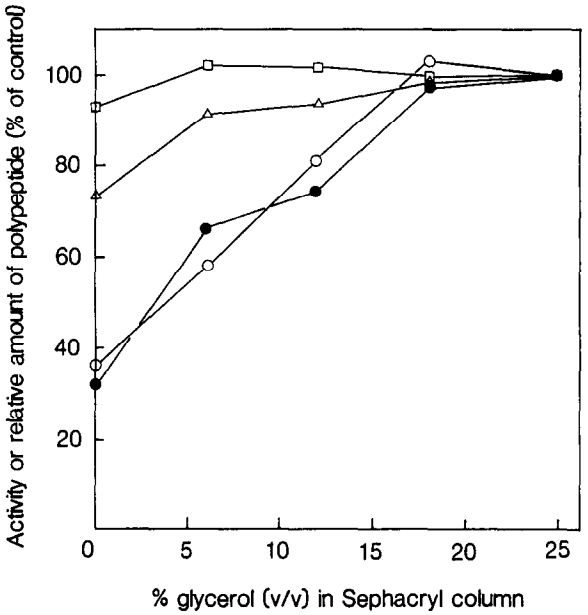

Fig.2. Effect of treatment with 0–25% glycerol on oxygen evolution and the relative amounts of 3 polypeptides in *P. laminosum* PS II particles. Oxygen evolution (●) was assayed at pH 6.0 with 10 mM ferricyanide as acceptor in 40 mM Mes plus 25% glycerol and 10 mM $CaCl_2$. Control (100%) activity, for PS II particles treated with 25% glycerol, corresponded to 2063 μmol O_2/mg chl *a* per h. LiDS-urea-PAGE was carried out at 4°C in gels containing 0.1% LiDS and 4 M urea. Each lane was scanned densitometrically, and relative amounts of the 9 kDa (○), 30.5 kDa (△) and 33 kDa (□) polypeptides calculated, normalizing against the amount of a 7 kDa polypeptide (cytochrome *b*-559) that was unaffected by glycerol concentration. Control (100%) amounts of the polypeptides were taken as the amounts present in PS II particles treated with 25% glycerol.

(not shown) and of the 30.5 and 33 kDa polypeptides (fig.2) were insufficient to account for the activity loss. These results support the contention that a major site for the glycerol effect on oxygen evolution is the 9 kDa polypeptide.

The 9 kDa polypeptide has been shown to participate in PS II activity on the oxidizing side of the reaction centre [1], but its exact function is still unknown. It does not appear to be associated with manganese: we have shown previously that inactivating PS II preparations from *P. laminosum* by low-glycerol treatment did not have a significant effect on their manganese content [7], nor did removal of the 9 kDa polypeptide by 1 M salts [1]. Nor is the 9 kDa polypeptide attributable to cytochrome *b*-559. Antibodies to spinach cytochrome *b*-559 have been found to react with a 7 kDa polypeptide in *P. laminosum* PS II particles and not with the 9 kDa polypeptide [1]. The 7 kDa polypeptide is not affected by glycerol (fig.1) or by high salt [1].

Table 1

Effect of salts on oxygen evolution in PS II particles treated with 2.5 or 25% glycerol

Salt present in assay medium	Oxygen evolution (μmol O_2/mg chl *a* per h)	
	2.5% glycerol-treated particles (50% 9 kDa polypeptide)	25% glycerol-treated particles (100% 9 kDa polypeptide)
None	484 (46)	891
1 mM $CaCl_2$	605 (50)	1214
5 mM $CaCl_2$	977 (46)	1814
10 mM $CaCl_2$	1050 (46)	1943
20 mM $CaCl_2$	1088 (46)	1900
10 mM $MgCl_2$	732 (43)	1404
20 mM NaCl	523 (46)	977
100 mM NaCl	455 (47)	852
5 mM $MnCl_2$	332 (61)	845
5 mM $Ca(Ac)_2$	551 (45)	1009

The figures in brackets show the degree of inhibition compared with 25% glycerol-treated PS II particles assayed under the same conditions. Particles were treated by Sephacryl S-300 chromatography as described in section 2. Activity was assayed in 40 mM Mes, pH 6.0, plus 25% glycerol, 10 mM ferricyanide and the salts shown

In higher plants, the extrinsic 23 and 16 kDa proteins participate in some way in increasing the affinity of PS II for Ca^{2+} and Cl^- [12–14]. Ca^{2+} and Cl^- are also required for oxygen evolution in cyanobacteria [15–17] but the 23 and 16 kDa proteins are lacking [1]. Our previous study indicated that the Ca^{2+} and Cl^- requirements in *P. laminosum* PS II particles were most marked when activity was assayed in the absence of glycerol, and therefore we speculated that the 9 kDa polypeptide might also be involved in the $CaCl_2$ effect. However, despite a 2-fold difference in the content of 9 kDa polypeptide, the activity of 2.5% glycerol-treated PS II particles as a proportion of that of control PS II particles remained approximately constant, regardless of the salts present in the assay medium (table 1). From double-reciprocal plots (not shown) it was calculated that the $CaCl_2$ concentration for half-maximal activity changed only slightly, from 0.7 mM (control) to 0.9 mM (depleted particles). Thus there does not seem to be any simple relationship between the 9 kDa polypeptide and the $CaCl_2$ requirement for oxygen evolution.

4. DISCUSSION

Our results have for the first time suggested a molecular basis for the glycerol/sucrose requirement for oxygen evolution in cyanobacterial preparations, by identifying the association between a 9 kDa polypeptide and PS II as a target for stabilization by glycerol in PS II particles from *P. laminosum*. However, conclusive proof of the importance of the 9 kDa polypeptide must await successful extraction/reconstitution experiments, and the protein is at present being purified with this in view.

The 9 kDa polypeptide does not appear to be functionally analogous to the 23 or 16 kDa proteins of higher-plant PS II, or to cytochrome *b*-559. However, a higher-plant protein that might possibly be related to the 9 kDa polypeptide of *P. laminosum* is an extrinsic polypeptide of 10 kDa that has recently been shown to be associated with spinach PS II [18]. Further experiments are needed to test this suggestion and also to determine whether other cyanobacteria contain a 9 kDa polypeptide similar to that of *P. laminosum*. Interestingly, a recent report has suggested that an active and highly resolved PS II preparation from the cyanobacterium *Synechococcus* sp. may contain, in addition to cytochrome *b*-559, other small polypeptide(s) of less than 10 kDa [19].

The fact that glycerol stabilizes association of the 9 kDa polypeptide with PS II indicates that hydrophobic forces contribute to the binding of the polypeptide, since high concentrations of polyols are known to promote hydrophobic protein-protein interactions [20]. In contrast, conditions which destabilize hydrophobic interactions, e.g. chaotropic salts, would be expected to remove the 9 kDa polypeptide and inhibit activity. This is consistent with the demonstrated removal of the 9 kDa polypeptide from *P. laminosum* PS II particles by 1 M chloride salts (NaCl, $MgCl_2$, $CaCl_2$), which at such high concentrations would exert a chaotropic effect [21].

Chaotropes lie towards one end of the Hofmeister or lyotropic series of salts. At the other extreme of this series are concentrated solutions of highly charged salts such as phosphate and citrate, which promote protein-protein interactions [21]. We have found that phosphate and citrate, like glycerol, stabilize activity and binding of the 9 kDa polypeptide in PS II preparations from *P. laminosum* (Stewart, unpublished). It is striking that these conditions also act in vitro to promote association of the light-harvesting phycobilisomes with PS II [22]. Thus hydrophobic interactions appear to be of general importance in association of extrinsic proteins with the PS II core in cyanobacteria, though how these associations are maintained in vivo remains to be elucidated.

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REFERENCES

[1] Stewart, A.C., Ljungberg, U., Åkerlund, H.-E. and Andersson, B. (1985) Biochim. Biophys. Acta 808, 353–362.

[2] Åkerlund, H.-E. (1983) in: The Oxygen-Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp.201–208, Academic Press Japan, Tokyo.
[3] Thomas, J.B. and De Rover, W. (1955) Biochim. Biophys. Acta 16, 391–395.
[4] Fredericks, W.W. and Jagendorf, A.T. (1964) Arch. Biochem. Biophys. 104, 39–49.
[5] Tel-Or, E. and Avron, M. (1974) in: Proceedings of the 3rd International Congress on Photosynthesis (Avron, M. ed.) pp.569–578, Elsevier, Amsterdam, New York.
[6] Stewart, A.C. and Bendall, D.S. (1980) Biochem. J. 188, 351–361.
[7] Stewart, A.C. and Bendall, D.S. (1981) Biochem. J. 194, 877–887.
[8] Satoh, K. and Katoh, S. (1985) Biochim. Biophys. Acta 806, 221–229.
[9] Bowes, J.M., Stewart, A.C. and Bendall, D.S. (1983) Biochim. Biophys. Acta 725, 210–219.
[10] Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y. and Wada, K. (1974) Biochim. Biophys. Acta 357, 231–245.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130.
[13] Andersson, B., Critchley, C., Ryrie, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. 168, 113–117.
[14] Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120.
[15] Piccioni, R.G. and Mauzerall, D.C. (1978) Biochim. Biophys. Acta 504, 384–397.
[16] Brand, J.J. (1979) FEBS Lett. 103, 114–117.
[17] England, R.R. and Evans, E.H. (1983) Biochem. J. 210, 473–476.
[18] Ljungberg, U., Åkerlund, H.-E. and Andersson, B. (1984) FEBS Lett. 175, 255–258.
[19] Satoh, K., Ohno, T. and Katoh, S. (1985) FEBS Lett. 180, 326–330.
[20] Gekko, K. and Timasheff, N. (1981) Biochemistry 20, 4667–4676.
[21] Jencks, W.P. (1969) in: Catalysis in Chemistry and Enzymology, pp.351–392, McGraw-Hill, New York.
[22] Katoh, T. and Gantt, E. (1979) Biochim. Biophys. Acta 546, 383–393.