Ca^{2+} requirement for photosynthetic oxygen evolution of spinach and mangrove photosystem II membrane preparations

Christopher Preston and Christa Critchley*

Botany Department, The Faculties, Australian National University, GPO Box 4, Canberra, ACT 2601, Australia

Received 22 March 1985

$CaCl_2$ significantly increased the photosynthetic oxygen-evolution activity of photosystem II membrane preparations isolated from spinach and the mangrove *Avicennia marina*, but not that of spinach thylakoids. This increase is composed of two parts; one due to Cl^-, the other to Ca^{2+}. Under Cl^--sufficient conditions photosystem II particle preparations required only 2 mM Ca^{2+} for maximum oxygen evolution, irrespective of the presence of the 23- and 18-kDa polypeptides. It is suggested that there is no immediate interaction between Ca^{2+} and the 23- or 18-kDa polypeptides and that instead Ca^{2+} acts directly on the oxygen-evolving centre, perhaps via another, as yet unidentified protein.

Calcium *Photosynthetic oxygen evolution* *Oxygen-evolving protein* *Chloride* *Salt-washing*

1. INTRODUCTION

Three polypeptides of 33, 23 and 16–18 kDa located at the inner thylakoid surface have been shown to be components of the photosynthetic oxygen-evolving system [1–3]. Many recent studies have concentrated on depleting PS II preparations of these proteins and found a corresponding loss of O_2-evolving activity, which was reversible by reconstituting activity after readdition of these proteins [3–8]. Recently, it has also been shown that Cl^- can reactivate preparations depleted of 23- and 18-kDa proteins [9,10] and additionally, that the 23-kDa protein modulates the Cl^- requirement. The same has been claimed for Ca^{2+} [10–12], which was also able to reconstitute activity in membranes depleted of the 33-kDa protein [13].

We present evidence here that the Ca^{2+} requirement for oxygen evolution is altered neither by the absence of the 23- and 18-kDa polypeptides in either spinach or mangrove membranes, nor by reconstitution of these polypeptides, and that only the magnitude of the Ca^{2+} response is changed. We also show that the response mediated by $CaCl_2$ is partly due to Cl^- and that only 2 mM Ca^{2+} is required for optimal activity in both membrane systems.

2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) was grown in vermiculite and watered daily with nutrient solution. Mangrove leaves (*Avicennia marina*) were collected from a mangrove swamp on Cullendulla Creek, Batemans Bay, Australia.

Spinach thylakoids and PS II particles were prepared as in [14] and [15], respectively. The second detergent wash was replaced by a wash in

* To whom correspondence should be addressed

Abbreviations: AMPD, 2-amino-2-methyl-1,3-propanediol; chl, chlorophyll; FeCN, ferricyanide; LiDS-PAGE, lithium dodecyl sulphate-polyacrylamide gel electrophoresis; Mes, 4-morpholinoethanesulphonic acid; OEP, oxygen-evolving particle; PBQ, phenyl-*p*-benzoquinone; PS II, photosystem II

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 40 mM Mes-AMPD, pH 6.5, and particles were resuspended in the same buffer. Mangrove thylakoids were prepared as described in [16] except that the wash buffer comprised 50 mM Hepes-AMPD, pH 7.3, and the final resuspension buffer was 300 mM sorbitol, 10 mM NaCl, 10 mM $MgCl_2$ and 40 mM Mes-AMPD, pH 6.0. Mangrove oxygen-evolving particles were prepared by incubating thylakoids at 1.5 mg chl/ml with 25:1 Triton X-100:chl for 5 min. This incubation mixture was centrifuged at 40000 × *g* for 30 min and washed as described for spinach PS II particles. The mangrove thylakoids (and OEP) prepared in this way are routinely devoid of the 23- and 18-kDa polypeptides [9,16].

Salt-washing of spinach PS II particles was performed by resuspending particles at 1 mg chl/ml in 1 M NaCl, 300 mM sorbitol and 40 mM Mes-AMPD, pH 5.4, stirring for 30 min at 4°C. Particles were then centrifuged (40000 × *g*, 20 min), the supernatant collected and the pellet washed in 300 mM sorbitol, 10 mM NaCl and 40 mM Mes-AMPD, pH 6.5, and stored on ice in the same solution. The supernatant was dialysed against a solution of 20 mM Mes-AMPD, pH 6.5, for 1.5 h with a 500:1 ratio of dialysis medium to sample, followed by 30 min on a bed of dry sucrose to concentrate the protein. Reconstitution was achieved by adding 1 mg protein to 1 mg chl allowed to aggregate for 30 min and then centrifuged in an Eppendorf centrifuge for 5 min. The reconstituted particles were resuspended in the same medium as the washed particles.

Oxygen-evolution activity was measured in an O_2 electrode, illuminated with saturating red light using 25 mM Mes-AMPD, pH 6.5, as the basic assay buffer with 0.5 mM FeCN and 0.13 mM PBQ as electron acceptors and 2.5 mM NH_4Cl as an uncoupler where required. The final volume was 3 ml with 10 mg chl/ml. NaCl and $CaCl_2$ concentrations are given in the figure legends. Chlorophyll was determined as in [17]. LiDS-PAGE was performed as described in [18].

3. RESULTS

Spinach thylakoids showed a slight increase in activity with the addition of NaCl and $CaCl_2$ (fig.1), and there was little difference in the effects of the two salts. By contrast, $CaCl_2$ caused a large (64%) stimulation of oxygen evolution in spinach PS II particles, whereas NaCl only stimulated activity by half as much. Removal of the 23- and 18-kDa polypeptides by salt-washing caused a substantial inhibition of oxygen evolution which could be partly reversed by the addition of NaCl and to a much greater extent by addition of $CaCl_2$. NaCl could only increase oxygen evolution by 50% of that observed with $CaCl_2$. Reconstituting salt-washed particles by adding back the 23- and 18-kDa polypeptides resulted in an increase in oxygen evolution in the absence of Cl^-. The addition of Cl^- did not increase activity by as much as in the non-reconstituted particles and again $CaCl_2$ was able to increase activity by twice as much as NaCl.

Mangrove thylakoids showed a much greater increase in activity with the addition of Cl^- than did the spinach thylakoids, with $CaCl_2$ giving a greater increase than NaCl (fig.2). Mangrove oxygen-evolving particles had very low activity in the absence of added Cl^- which increased upon addition of Cl^- to levels of activity similar to those of the thylakoids, indicating a large loss of Cl^- during

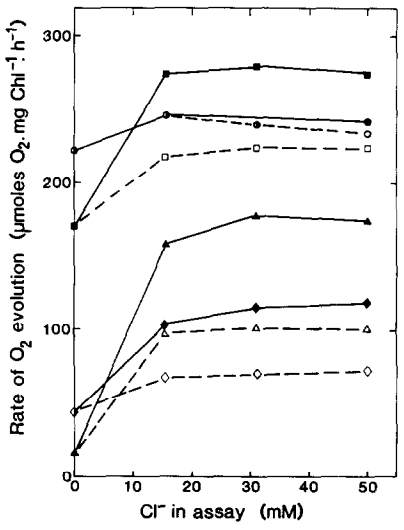

Fig.1. O_2-evolution activity as a function of NaCl and $CaCl_2$ in spinach thylakoids (●—●); PS II particles (■—■); salt-washed PS II particles (▲—▲); and salt-washed PS II particles reconstituted with dialysed and concentrated supernatant (◆—◆). NaCl, open symbols; $CaCl_2$, solid symbols.

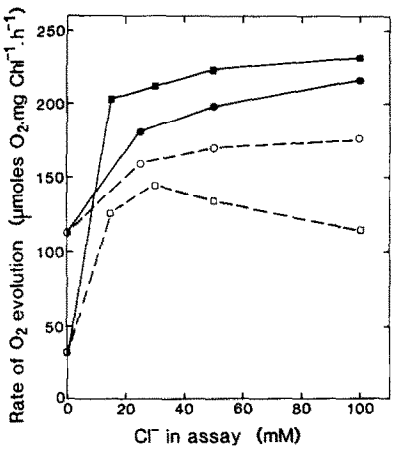

Fig.2. O_2-evolution activity of mangrove thylakoids (●—●) and OEP (■—■) as influenced by NaCl (open symbols) or $CaCl_2$ (solid symbols) concentrations.

isolation. With these particles $CaCl_2$ was able to increase activity by nearly twice as much as NaCl.

The amount of Ca^{2+} required for oxygen evolution was found to be very low once the particles were supplied with sufficient Cl^-. This value was found to be 2 mM and was independent of the presence or absence of the 23- and 18-kDa polypeptides (fig.3). In these experiments the Cl^- concentration was kept constant, i.e., 32 mM for spinach PS II particles (fig.3A) and 100 mM for mangrove membranes (fig.3B), and Ca^{2+} varied. Salt-washing or reconstituting salt-washed particles did not alter the amount of Ca^{2+} required, although it did change the magnitude of the response (fig.3A). Ca^{2+} produced the largest increase in activity with salt-washed spinach PS II particles and mangrove oxygen-evolving particles, both of which are devoid of the 23- and 18-kDa polypeptides (fig.4, [9,16]). The increases due to Ca^{2+} with spinach PS II particles and reconstituted particles were less.

LiDS-PAGE showed the effect of salt-washing and reconstitution of spinach PS II particles on their polypeptide compositions (fig.4). Salt

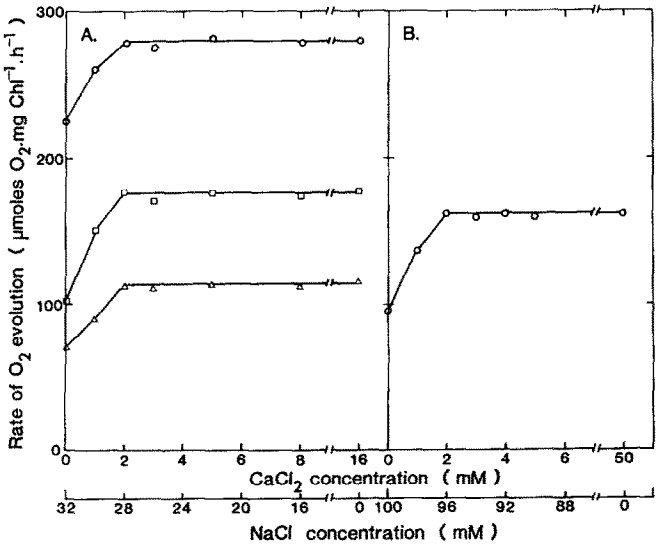

Fig.3. Ca^{2+} requirement for oxygen evolution in spinach PS II particles (A) and mangrove OEP (B). Control particles (○—○); salt-washed particles (□—□); and reconstituted salt-washed particles (△—△).

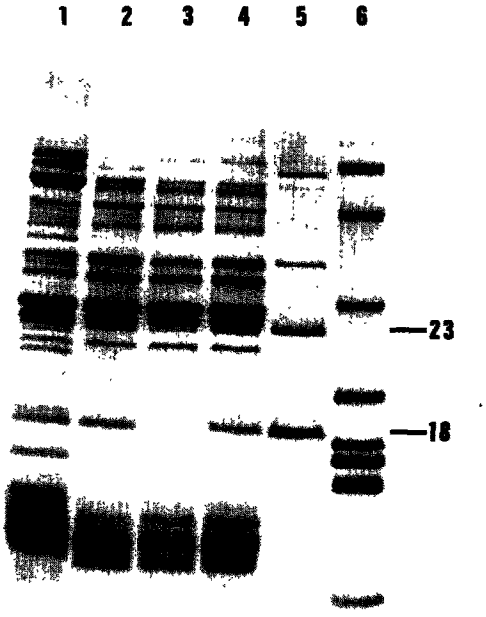

Fig.4. LiDS-PAGE of proteins of spinach thylakoids and PS II particles. Lanes: 1, spinach thylakoids; 2, spinach PS II particles; 3, salt-washed spinach PS II particles; 4, salt-washed spinach PS II particles reconstituted with dialysed and concentrated supernatant; 5, supernatant from salt-washed spinach PS II particles; 6, molecular mass markers: β-galactosidase, 130 kDa; phosphorylase *a*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20.1 kDa; myoglobin, 17.2 kDa; lysozyme, 14.3 kDa; cytochrome *c*, 11.7 kDa; and insulin, 5.7 kDa. The positions of the 23- and 18-kDa polypeptides are indicated.

washing removed the 23- and 18-kDa polypeptides (lanes 3,5), which could rebind after reconstitution (lane 4). Salt washing also removed two other polypeptides of 35 and 65 kDa which are not involved in oxygen evolution (lane 5). Western blotting confirmed that the salt-washed spinach particles and the mangrove membranes had lost the 23- and 18-kDa proteins (not shown, but see [16]).

4. DISCUSSION

Several recent studies [10–12,19,20] have investigated the involvement of Ca^{2+} in photosynthetic oxygen evolution. These studies have concluded that Ca^{2+} is an essential cofactor for oxygen evolution and interacts strongly with the 23- and 18-kDa polypeptides (see [11,19]). This study confirms the role of Ca^{2+} in oxygen evolution, but indicates that there may not be any direct interaction between Ca^{2+} and these polypeptides. Ca^{2+} was able to increase oxygen evolution activity in all PS II particle preparations regardless of the presence of 23- and 18-kDa proteins, but not in spinach thylakoids (fig.1). This would indicate that the large Ca^{2+} cannot gain access to the site of oxygen evolution in the sealed thylakoids whereas it can in the open PS II particles. Mangrove thylakoids must be somewhat leaky as Ca^{2+} can increase oxygen evolution significantly (fig.2). This is not surprising as these membranes are uncoupled and electron transport between the two photosystems is interrupted [21], indicating a degree of damage during isolation.

In all cases where $CaCl_2$ increased oxygen evolution in PS II particles this was shown to be composed of an increase due to Cl^-, and superimposed on this was an increase due to Ca^{2+}. This has not always been recognised in earlier studies. Miyao and Murata [12] found no increase in oxygen evolution due to NaCl and little due to $CaCl_2$ in PS II particles, however, their basic assay buffer contained 10 mM NaCl which is nearly sufficient to saturate oxygen evolution by spinach PS II particles (see fig.1). Ghanotakis et al. [11] did not even consider possible effects of Cl^- in their study.

Here we investigated the Ca^{2+} requirement for oxygen evolution under Cl^- sufficient conditions and found a requirement of 2 mM Ca^{2+} irrespective of the presence of the 23- and 18-kDa polypeptides, or whether membranes were isolated from spinach or the halophyte *Avicennia marina* (fig.3). This clearly indicates that the 23- and 18-kDa proteins do not act to concentrate Ca^{2+}. Ghanotakis et al. [19] reported that the 23- and 18-kDa proteins reduced the Ca^{2+} requirement; however, they did not control Cl^- in their assays, and it has been shown that the 23-kDa protein increases the affinity of the water oxidation site for Cl^- [9].

The single effect of removal of the 23- and 18-kDa proteins on the Ca^{2+}-induced increase in activity was to increase the magnitude of this stimulation. We propose that Ca^{2+}, by binding to carboxyl groups, may be able to alter the redox potential of the manganese centre to a more favourable value. Authors in [22] have already suggested that Ca^{2+} is able to alter the environment around the oxygen-evolving centre and a site sensitive to calcium antagonists has been demonstrated [20]. Authors in [23] have isolated a 13-kDa protein from PS II particles able to bind Ca^{2+} and this may be involved. The large increase in activity upon adding Ca^{2+} to salt-washed particles would be a result of the salt-washing altering the redox potential to a less favourable value and not to changes in the nature of the Ca^{2+} binding site. The site of Ca^{2+} action still has to be unequivocally determined and electron donor studies may help in localising the action to the manganese centre, Z or P680, or prove it to have a more delocalised effect.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Susan Young for performing the gel electrophoresis and Mrs Anne Gallagher for drawing the figures. C.P. and C.C. gratefully acknowledge support from a CPR award, and the Australian Government's National Research Fellowship Scheme, respectively.

REFERENCES

[1] Larsson, C., Jansson, C., Ljungberg, U., Akerlund, H.-E. and Andersson, B. (1984) in: Advances in Photosynthesis Research, vol.I (Sybesma, C. ed.) pp.363–366, Martinus Nijhoff/W. Junk, The Hague.

[2] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.

[3] Akerlund, H.-E. (1983) in: The Oxygen Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp.201–208, Academic Press, Tokyo.
[4] Ono, T. and Inoue, Y. (1984) FEBS Lett. 166, 381–384.
[5] Ljungberg, U., Jansson, C., Andersson, B. and Akerlund, H.-E. (1983) Biochem. Biophys. Res. Commun. 113, 738–744.
[6] Akabori, K., Imaoka, A. and Toyoshima, Y. (1984) FEBS Lett. 173, 36–40.
[7] Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93.
[8] Miyao, M. and Murata, N. (1983) FEBS Lett. 164, 375–378.
[9] Andersson, B., Critchley, C., Ryrie, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. 168, 113–117.
[10] Nakatani, H.Y. (1984) Biochem. Biophys. Res. Commun. 120, 299–304.
[11] Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130.
[12] Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120.
[13] Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286.
[14] Robinson, H.H. and Yocum, C.F. (1980) Biochim. Biophys. Acta 590, 97–106.
[15] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[16] Critchley, C., Andersson, B., Ryrie, I.J. and Anderson, J.M. (1984) Biochim. Biophys. Acta, in press.
[17] Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
[18] Ryrie, I.J. (1983) Eur. J. Biochem. 131, 149–155.
[19] Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173.
[20] Nakatani, H.Y. (1984) Biochem. Biophys. Res. Commun. 121, 626–633.
[21] Critchley, C. (1982) Nature 298, 483–485.
[22] Yerkes, C.T. and Babcock, G.T. (1981) Biochim. Biophys. Acta 634, 19–29.
[23] Sparrow, R.W. and England, R.R. (1984) FEBS Lett. 177, 95–98.