The release of polypeptides and manganese from oxygen-evolving photosystem II preparations following zinc-treatment

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Inhibition of oxygen evolution in photosystem II membrane fragments from pea chloroplasts by washing with Zn^{2+} causes appearance of the EPR signal of $Mn(H_2O)_6^{2+}$. This Mn^{2+} remains associated with the membrane fraction. Release of Mn^{2+} into the medium was correlated with the amount of the 23 kDa protein removed from the membrane. This suggests that this protein may function as a 'gate' to an aqueous compartment into which Mn^{2+} is released. Inhibition by Zn^{2+} correlated with the release of 1 Mn^{2+} per reaction centre, out of a total stoichiometry of 4 Mn atoms per reaction centre. By comparing the release of Mn following Zn-treatment of NaCl or $CaCl_2$ washed membranes, it is concluded that the 33 kDa protein is involved in binding of 2 Mn.

Photosystem II *Oxygen evolution* *Manganese* *Polypeptide*

1. INTRODUCTION

The involvement of manganese in photosynthetic oxygen evolution has been known for many years, but there is still uncertainty about its stoichiometry and the mechanism of its participation in the water-splitting process [1]. Recent investigations on membrane preparations enriched in photosystem II have shown that 3 polypeptides of around 33, 23 and 16 kDa are associated with the photosynthetic oxygen evolution process [2]. Immunological studies revealed that the 3 proteins form a complex at the inner thylakoid surface. The 33 kDa protein is required for binding of the 23 kDa protein which in its turn enhances binding of the 16 kDa protein [3].

There are contradicting views on the function of these proteins. It has been suggested that the 33 kDa protein is the manganese binding protein [4,5], although the protein can be removed from photosystem II membrane fragments without affecting the Mn content [6]. The 23 kDa protein has been claimed to have a regulatory function [7,8] and reported by [9] to play an obligatory role in the oxygen evolution process. The 16 kDa protein does not seem to play a vital role; no correlation was found between the amount of this protein and oxygen evolution activity [10]. The two smaller proteins have been implicated in binding of ions involved in the water-splitting process: the 23 kDa protein promotes a high affinity binding of Ca^{2+} [11] and is able to lower the concentration of chloride required for optimal oxygen evolution activity [12]. A role as a Cl^--binding protein has also been proposed for the 16 kDa protein [13].

Manganese released from thylakoid membranes upon inhibition of oxygen evolution by treatment with alkaline Tris buffer [14] or Zn^{2+} [15] has the same EPR spectrum as free hexaquo $Mn(H_2O)_6^{2+}$. This implies its occurrence in an aqueous compartment originally equated by Blankenship and Sauer [14] with the thylakoid lumen. However, we observed that the released Mn^{2+} remained associated with the membrane fraction upon centrifugation even in non-vesicular photosystem II preparations [16]. To resolve this apparent paradox, we

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suggested that the various proteins of the photosystem II complex might be arranged around a central aqueous compartment into which Mn was released following the inhibitory treatments [16]. To examine this possibility further, we have compared the release of polypeptides and manganese from oxygen-evolving photosystem II membrane fragments following various inhibitory treatments.

2. MATERIALS AND METHODS

Peas were grown in vermiculite under artificial light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 21–23°C. Oxygen-evolving photosystem II membrane fragments were prepared from pea thylakoids by treatment with Triton X-100 in the presence of $MgCl_2$ using the method of Berthold et al. [17] as modified in [18]. The chlorophyll concentration was determined as in [19].

Manganese was determined by EPR measurements as in [20]. Total Mn^{2+} was determined following acidification with HCl to a final concentration of 0.2 M HCl. Quantitative values for the concentration of Mn were always determined using standards in identical media.

Cytochrome *b*-559 was determined using a Cary 118c spectrophotometer as described in [21]. The membrane fragments were suspended in 50 mM Mops-NaOH (pH 6.8) at 50 μg chl/ml. Total cytochrome *b*-559 was determined from dithionite reduced minus ferricyanide oxidized difference spectra. A value of 15 $mM^{-1} \cdot cm^{-1}$ was used as the difference extinction coefficient.

Oxygen evolution with 0.5 mM phenyl-*p*-benzoquinone as photosystem II acceptor was measured polarographically at 22°C in a medium containing 10% glycerol, 5 mM $MgCl_2$, 20 mM Mes (pH 6.0) and membrane fragments corresponding to 20 μg chl/ml.

SDS-polyacrylamide gel electrophoresis was run in the buffer system of Laemmli [22] using slab gels containing a 12–20% acrylamide gradient. Prior to solubilization for 5 min at 4°C in 2% SDS, 5% mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, the samples were dialyzed overnight against reaction medium to minimize the concentration of divalent salts. The gels were stained with Coomassie brilliant blue R-250. For densitometric quantitation the proteins were measured at 663 nm with a soft laser scanning densitometer (Biomed Instruments). The relative amounts of the various polypeptides were estimated from the peak heights of the stained bands in the densitogram.

Inhibitory treatments of the photosystem II preparations were all done in the dark at 4°C in a reaction medium composed of 0.33 M sorbitol, 20 mM Hepes-NaOH, pH 7.0.

$CaCl_2$-washing was performed essentially according to [6]. Membranes (0.5 mg chl/ml) were incubated for 30 min in reaction medium containing 1 M $CaCl_2$. The suspension was then diluted to 50 μg chl/ml with the reaction medium. After centrifugation at 27 000 × *g* for 20 min the pellet was resuspended in a small volume of reaction medium.

For NaCl treatment the photosystem II preparation was diluted to 50 μg chl/ml in reaction medium containing 250 mM NaCl. After incubation for 30 min the suspension was centrifuged at 27 000 × *g* for 20 min. The pellet was then resuspended in reaction medium.

For Zn^{2+} treatment membranes corresponding to 0.2 mg chl/ml were incubated in reaction medium containing 5 mM $ZnSO_4$ unless otherwise indicated. Immediately prior to centrifugation (18 000 × *g*, 4 min) the polycation polybrene was added (final concentrations 25–35 μg/ml) to allow rapid and complete precipitation of the membrane fragments. When peptides were to be measured by SDS-PAGE, polybrene was omitted and centrifugation was at 27 000 × *g* for 5 min. The pellet was resuspended in a volume of the supernatant corresponding to 10% of the original.

The ratio of chlorophyll to photosystem II reaction centres was estimated by two independent methods; firstly from the total cytochrome *b*-559 content (assuming a stoichiometry of 2 cytochromes per reaction centre) and secondly from the total amount of O_2 produced by a sequence of saturating single turnover flashes. In this case the flash had 90% of its integrated intensity within 80 μs and the results obtained at 20 and 1°C were very similar, confirming that the number of double turnovers was small under these conditions.

3. RESULTS

3.1. *Relationship between polypeptide composition and manganese compartmentation*

We have previously shown that the photosyn-

thetic oxygen-evolution process in sub-chloroplast preparations is inhibited by low concentrations of Zn^{2+} [16]. Fig.1 shows that the Zn-inhibition of oxygen-evolution in photosystem II preparations can be correlated with the conversion of endogenous manganese to the EPR-detectable form.

Fig.2 shows that during the Zn^{2+} treatment, the 16 and 23 kDa proteins were progressively removed from the membranes. The 33 kDa protein was not significantly released even after incubation for 50 min. This pattern of release confirms the idea that the 16 and 23 kDa proteins are bound to the photosystem II protein complex by weak electrostatic forces, in contrast to the 33 kDa protein, which is thought to be attached by hydrogen bonding [3].

Release of the 16 kDa protein was too fast for the kinetics to be followed under these conditions (not shown). Fig.3 shows that the release of the 23 kDa polypeptide occurred more slowly than the inhibition of the oxygen evolution process: when the oxygen evolution was less than 10% of the original activity, more than 50% of the 23 kDa protein was still associated with the membrane fraction. This suggests that the inhibition does not result directly from release of this polypeptide. After 60 min incubation, 15% of the 23 kDa protein is still apparently associated with the membrane fraction. This is at least in part a consequence of the experimental procedure adopted, in which the membrane fractions were resuspended in a small volume of their own supernatant.

The EPR-detectable manganese released from

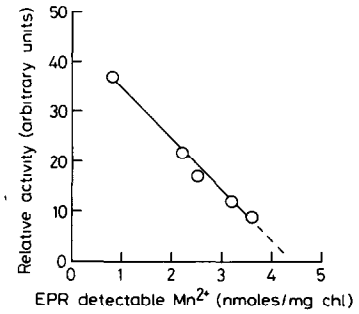

Fig.1. Relationship between EPR-detectable Mn^{2+} and oxygen-evolution activity in photosystem II membrane fragments following treatment with various [$ZnSO_4$] for 2 min. Sample preparation was performed as described in section 2. Control activity: 240 μmol O_2 · (mg chl)$^{-1}$ · h^{-1}.

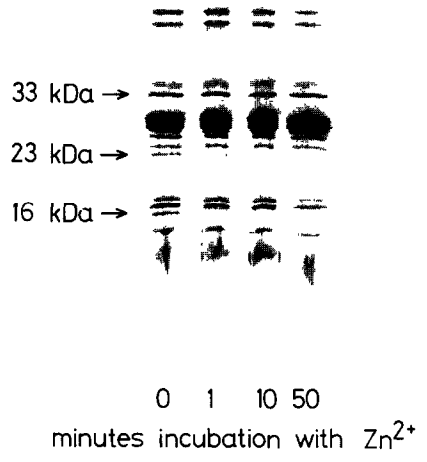

Fig.2. Polypeptide profiles of photosystem II membrane fragments after treatment with 5 mM $ZnSO_4$. Samples were taken at the times indicated and after centrifugation the pellets were resuspended and prepared for electrophoresis, as described in section 2. The locations of the bands corresponding to 33, 23 and 16 kDa are indicated.

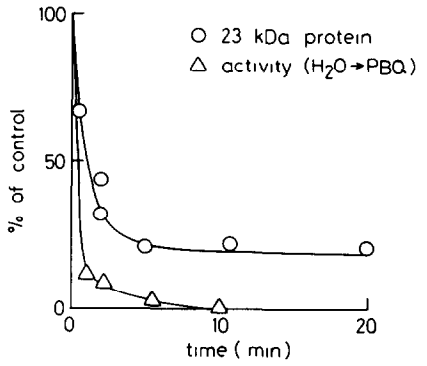

Fig.3. Inhibition of oxygen-evolution and release of the 23 kDa protein from photosystem II membrane fragments following Zn-treatment. Following incubation for the times shown the membranes were separated by centrifugation without polybrene, so the effective incubation times are about 10 min longer. Oxygen-evolution activity and polypeptide composition were determined on the resuspended pellets as described in section 2.

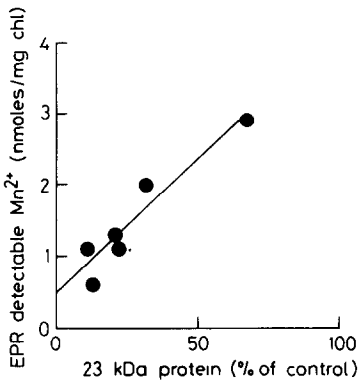

Fig.4. Correlation between EPR-detectable Mn^{2+} and the amount of 23 kDa protein bound to Zn-treated membrane fragments. Photosystem II membrane fragments (0.1 mg chl/ml) were treated with 5 mM $ZnSO_4$ for various times (between 0.5 and 50 min), and EPR-detectable manganese and the polypeptide composition determined as in section 2.

photosystem II preparations after Zn-treatment is retained in the membrane fraction and equilibration with the surrounding medium takes several hours with thylakoids [15]. For photosystem II membrane fragments the loss of Mn^{2+} was somewhat faster and more variable between preparations. As illustrated in fig.4 the efflux of EPR-detectable manganese from the membrane fraction can be correlated with the release of the 23 kDa protein. Thus if release of Mn^{2+} is into a restricted compartment, the 23 kDa protein might function as a gate.

As a test of this idea, Zn-treatments were performed on photosystem II membranes already depleted of the 23 kDa protein by washing with NaCl or $CaCl_2$. High concentrations of NaCl inhibit O_2 evolution and release the 16 and 23 kDa proteins without affecting the manganese content of the membrane [10]. Further treatment of the NaCl-washed membranes with 5 mM Zn^{2+} resulted in complete inhibition of photosynthetic oxygen evolution and a rapid decrease in the Mn content (table 1). The concentration of $Mn(H_2O)_6^{2+}$ in the membrane fraction did not exceed that measured in the supernatant indicating that the released Mn was in immediate equilibrium with the surrounding medium (not shown).

Washing the photosystem II membrane preparation with 1 M $CaCl_2$ is an inhibitory treatment which was found to liberate the 16, 23 and 33 kDa proteins without affecting the Mn content of the

Table 1

The effect of various treatments on photosynthetic activity and manganese release in oxygen-evolving photosystem II preparations

Treatment	Activity [μmol $O_2 \cdot$ (mg chl)$^{-1} \cdot h^{-1}$]	Total Mn (nmol/mg chl)	Mn/RC[a]	Difference
Control	233	17.8	4.6	
NaCl	105	16.5	4.3	0.9
NaCl, $ZnSO_4$	6	13.0	3.4	
$CaCl_2$	0	16.2	4.2	2.6
$CaCl_2$, $ZnSO_4$	0	6.3	2.6	
Heat[b]	0	2.4	0.6	
pH 9.3[c]	0	2.3	0.6	

[a] Calculations based on a value of 290 chlorophyll per reaction centre of photosystem II. RC = reaction centre

[b] Membrane fragments (0.4 mg chl/ml) were incubated in reaction medium containing 20 mM $MgCl_2$ in the dark for 10 min at 45°C. After centrifugation (30 min, 27 000 × g) the pellet was resuspended in a volume of the supernatant corresponding to 10% of the original

[c] Membrane fragments (0.4 mg chl/ml) were incubated for 10 min at 0°C in the dark in a medium containing 0.33 M sorbitol, 20 mM $MgCl_2$, 25 mM Taps-NaOH, pH 9.3. Centrifugation and resuspension were as described above

membranes, confirming the report in [6]. When the membranes were prewashed with 1 M $CaCl_2$ and then treated with $ZnSO_4$ 9.9 nmol Mn/mg chl were released (table 1). Again, after Zn-treatment, the concentration of EPR-detectable Mn in the membrane fraction was equal to that found in the supernatant (not shown). Thus with both the NaCl and $CaCl_2$ treatments, prior removal of the 23 kDa protein caused immediate release of manganese from the membranes, providing further support for the idea of a 'gating' role for this peptide.

3.2. *Stoichiometry of manganese released from photosystem II preparations*

It has become clear that the ratio between chlorophyll and reaction centres can vary considerably depending on the conditions under which plants are grown [23]. The plant material used in this study was grown under relatively weak light. From the cytochrome *b*-559 content of the membrane fragments (6.8 nmol/mg chl) the antenna size of the photosystem II reaction centre was estimated as 290 chl assuming 2 *b*-559 per reaction centre [24]. The same value was obtained from measurements of O_2 produced during single turnover flashes (see section 2). The total manganese content in the oxygen-evolving photosystem II preparations used in this study was 17.8 nmol/mg chl (table 1). This amount was not decreased by addition of 5 mM $MgCl_2$, indicating that the photosystem II preparation does not contain any unspecifically associated manganese (not shown). Any Mn^{2+} previously loosely associated with the membrane fragments would be replaced by Mg^{2+} during the isolation procedure.

This total manganese content corresponds to 4.6 Mn per photosystem II reaction centre. It comprises both that known to be functional in the water oxidation process, and released by suitable inhibitory treatments, and also a manganese pool which is more difficult to remove from the membranes. Some of this very strongly bound manganese may be associated with the light harvesting chlorophyll *a/b* protein complex [1]. After incubation at pH 9.3 or heating at 45°C for 10 min in the presence of 20 mM $MgCl_2$ most of the manganese was removed. The residual manganese (table 1) corresponded to 0.6 Mn per reaction centre, suggesting a stoichiometry of 4 Mn removed by these extreme treatments. This supports the idea that 4 Mn occur in each oxygen-evolving complex. By extrapolation of the results shown in fig.1 it is found that loss of one of the 4 Mn atoms after Zn-treatment leads to a complete inactivation of the oxygen-evolution. We reported [15] that full inhibition of the oxygen-evolution in thylakoids isolated from lettuce chloroplasts by Zn-treatment corresponded to release of 2 Mn atoms per reaction centre of photosystem II. The reason for this discrepancy is not clear but may originate from the different membrane structure and composition of the photosystem II preparation and the lettuce thylakoids.

The question then arises as to whether it is possible to relate any of the 4 functional Mn to the various polypeptides of photosystem II reaction centre. As table 1 shows, Zn inhibition of NaCl-washed membranes leads to a release of one Mn/reaction centre. However, when the membranes had been pretreated with $CaCl_2$ to remove the 33 kDa protein in addition to the 16 and 23 kDa protein the amount of released Mn was increased to 2.6 Mn/reaction centre. This suggests that removal of the 33 kDa protein allows displacement of two Mn atoms, supporting the observations in [25].

4. DISCUSSION

The site of action of Zn^{2+} inhibition of the photosynthetic oxygen evolution is possibly associated with the ability of the Zn^{2+} to displace bound manganese involved in oxygen evolution [15]. The displaced manganese is retained in the membrane fraction as long as the 23 kDa protein is bound to the membranes. When the Zn-treatment was performed on preparations already depleted of the 16 and 23 kDa proteins the concentration of $Mn(H_2O)_6^{2+}$ in the membrane fraction did not exceed that found in the supernatant. These findings suggest that the 23 kDa protein may be involved in formation of an intermembranous compartment into which manganese is liberated following the inhibitory treatment with Zn. The compartment is apparently aqueous since the released manganese has the same spectral properties as free hexaqueous manganese [14].

The idea that the active site of the water-splitting enzyme resides in a special compartment is supported by the findings that H^+ [26] and Cl^- [27],

both associated with the oxygen-evolution process, can be trapped in compartments more restricted than the thylakoid lumen. Such a compartment could have a valuable function in shielding the charge accumulating complex against premature reduction by any internal or external reductants. Furthermore a compartment with a restricted permeability to ions could protect the active site of the water-splitting enzyme against fluctuations of the Ca^{2+} and Cl^- levels, and stabilise the binding of Mn to the enzyme by preventing the access of competitive ligands.

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