

Water-soluble 17 and 23 kDa polypeptides restore oxygen evolution activity by creating a high-affinity binding site for Ca^{2+} on the oxidizing side of Photosystem II

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Received 8 March 1984

Exposure of detergent-isolated preparations of the Photosystem II complex to 2 M NaCl releases water-soluble 17 and 23 kDa polypeptides; the inhibited rate of oxygen evolution activity is stimulated by addition of Ca^{2+} [(1984) FEBS Lett. 167, 127–130]. Reactivation of oxygen evolution activity by Ca^{2+} requires the presence of the ion in high (mM) non-physiological concentrations. Using a new dialysis-reconstitution procedure we have shown that rebinding of the 17 and 23 kDa polypeptides restores oxygen evolution activity only when the system has not been pretreated with EGTA. Removal of loosely-bound Ca^{2+} from the salt-extracted PS II complex and from the polypeptide solution, by dialysis against EGTA, blocks reconstitution of oxygen evolution activity even though the two polypeptides do rebind; restoration of Ca^{2+} to EGTA-treated systems, after rebinding of the 17 and 23 kDa polypeptides, results in a strong reconstitution of oxygen evolution activity. The effect of rebound 17 and 23 kDa polypeptides is to promote high affinity binding of Ca^{2+} to the reconstituted membrane.

Photosystem II Oxygen evolution Calcium Polypeptide

1. INTRODUCTION

A variety of investigations [1–3] have established that water-soluble 17 and 23 kDa polypeptides can be removed from inside-out thylakoid vesicles or PS II membranes prepared by detergent action. Loss of these polypeptides is correlated with a reduction in oxygen-evolution activity, although functional manganese is not perturbed by release of these polypeptides [3]. Authors in [1] first showed that addition of the 23 kDa polypeptide to salt-washed everted thylakoid vesicles would reconstitute some, but not all, of the oxygen evolution activity destroyed by polypeptide re-

moval. Addition of the 17 kDa species produced a much lower reconstitution activity. Since the 17 and 23 kDa polypeptides do not bind appreciable amounts of manganese, a structural role for these proteins in oxygen evolution has been proposed [1,3]. In [4], based on our earlier work with PS II membranes [3], we showed that preparations depleted of the 23 and 17 kDa polypeptides by exposure to 2 M NaCl at pH 6 displayed an oxygen evolving capacity 30–35% of the control activity. This activity could be restored to substantial levels (80% of control) by addition of Ca^{2+} . In [4], we were not in a position to assess the possible roles of Ca^{2+} and polypeptides in reactivating the OEC, and presented several hypotheses to explain our results. Here, we present the results of experiments using a new dialysis reconstitution technique for restoration of oxygen evolving activity. Our data show that water-soluble polypeptides and Ca^{2+} (at a high affinity binding site on the oxidizing side of

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Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; Mes, 4-morpholineethanesulfonic acid; PS, photosystem; OEC, oxygen evolving complex

PS II) are necessary components of the functional OEC.

2. MATERIALS AND METHODS

Preparation of the PS II complex was carried out as in [3,5] with the modification that the second Triton X-100 step was replaced by a wash with a pH 6 solution containing 400 mM sucrose, 15 mM NaCl and 50 mM Mes. The washed membranes were pelleted at $40000 \times g$ (30 min) and resuspended in the washing medium to a final concentration of 3 mg Chl/ml. Omission of the second Triton step resulted in a small PS I contamination and the oxygen evolution rates ($650\text{--}800 \mu\text{mol O}_2 \cdot \text{h}^{-1} \cdot \text{mg Chl}^{-1}$) are somewhat higher than in preparations isolated with two Triton exposures. Release of the 17 and 23 kDa polypeptides was carried out as in [3,4]. After

high-salt treatment the membranes were subjected to the various treatments described in section 3 to reconstitute oxygen evolution activity.

The oxygen evolution activity with 2,5-dichloro-*p*-benzoquinone as a PS II electron acceptor was followed using a Clark-type electrode and the polypeptide content of the membranes was examined by gel electrophoresis which was carried out as in [6] with the modification that 2.5 M urea was present in the gel and in the denaturing solutions.

3. RESULTS

Treatment of PS II membranes with 2 M NaCl results in removal of two water-soluble polypeptides with molecular masses of 17 and 23 kDa (fig.1, lane 2) [1–3]. High concentrations of NaCl apparently perturb the electrostatic interaction between the 23 and 17 kDa species and the rest of the PS II complex, but substantial activity can be restored to the depleted PS II complex upon addition of CaCl_2 to the assay medium [4]. This observation, along with several previous studies on



Fig.1. Gel electrophoresis patterns of: (1) control; (2) high-salt extracted membranes; (3) reconstituted PS II membranes. Reconstitution was carried out by resuspension of salt-washed PS II membranes in a fraction of the 2 M NaCl extract containing the 17 and 23 kDa polypeptides, followed by subsequent removal of NaCl by dialysis against a solution containing 0.4 M sucrose, 15 mM NaCl, 50 mM Mes (pH 6.0) (four 2-h steps, 4°C). After the last dialysis step the PS II membranes were pelleted by centrifugation, washed once with the pH 6.0 buffer used above, and resuspended in the same buffer. The amount of the polypeptides added back for the reconstitution experiments was usually equal to the amount removed by high-salt extraction. During dialysis the volume of the dialysis medium was one hundred times the volume of the sample in the dialysis bag. The gel contains 2 M urea. (It should be noted that there are two polypeptides in the 23 kDa region; one is the 23 kDa water-soluble polypeptide removed by high-salt treatment of the PS II complex and the other is a polypeptide, with higher molecular mass, which is not removed by either 2 M NaCl or even high pH (10); a better separation of the two polypeptides is observed when a higher concentration of urea is present during SDS gel electrophoresis.

cyanobacteria [7-9] and higher plant chloroplast systems [10-13], as well as a parallel study of the EPR signal of Z^+ (see [4]), implicate Ca^{2+} as a necessary cofactor in PS II catalyzed electron transfer. We have attempted to further examine the possible involvement of Ca^{2+} in photosynthetic oxygen evolution by removing Ca^{2+} from the native system (untreated complex) either by prolonged incubation with EGTA (\pm A23187) or by extensive dialysis against EGTA (\pm A23187); both treatments failed to inhibit oxygen evolution activity (table 1), which implies that if Ca^{2+} is required for activity it is very tightly bound.

Authors in [1] have developed a procedure for the reactivation of high-salt treated preparations of inside-out thylakoid vesicles which includes readdition of the desalted/concentrated polypeptide extract to the polypeptide depleted membranes. This method has also been applied to salt-extracted preparations of the PS II complex [2]. We have developed another protocol for reconstitution of oxygen evolution activity in salt-washed PS II membranes, which consists of combining the salt-washed PS II membranes with the high salt supernatant, containing the 17 and 23 kDa polypeptides, and then dialyzing this mixture to remove

NaCl. The details of this procedure are described in fig. 1, which presents an SDS polyacrylamide gel showing that the two polypeptides are rebound to the PS II membranes by this dialysis procedure (lane 3). As shown by the data of table 1, this dialysis procedure also restores high rates of oxygen evolution activity to the preparation, and this reconstituted activity is not inhibited by addition of EGTA and A23187. The fact that activity is insensitive to the chelator/ionophore combination, and the observation that activity does not require exogenous Ca^{2+} indicates that if Ca^{2+} is in fact required for activity, it is reincorporated into the PS II complex by this dialysis treatment, coincident with the rebinding of the two polypeptides.

In [4], we suggested that both water-soluble polypeptides and Ca^{2+} were required for high rates of oxygen evolution activity, and to further explore this possibility we have used variations on the dialysis reconstitution technique to examine the role of Ca^{2+} in PS II. In these experiments, we first expose both the salt-washed PS II membranes and the high-salt extract to dialysis against EGTA to remove any loosely-bound Ca^{2+} that may be present in either fraction. Following this treatment, the two fractions are recombined, and NaCl is removed by dialysis under conditions whereby Ca^{2+} is either excluded or included in the reconstitution experiment. The results of representative studies are shown in table 2. As these data clearly illustrate, the reactivation of the PS II complex to a state where high rates of oxygen evolution activity are observed is necessarily dependent on the inclusion of polypeptides during dialysis, as well as a dialysis step in which Ca^{2+} is added to the complex. For the experiments shown, polypeptide binding to the PS II complex was observed regardless of whether Ca^{2+} was present or not (not shown).

As can be seen in table 2, rebinding of polypeptides under conditions which rigidly exclude the presence of loosely bound Ca^{2+} does not produce a restoration of high rates of oxygen evolution activity. We have further examined this preparation, to which the polypeptides are rebound, to see whether we could restore oxygen evolution activity by addition of Ca^{2+} . Fig. 2 summarizes the results of experiments comparing PS II membranes with and without the rebound polypeptides to which Ca^{2+} was added; after the incubation times shown,

Table 1

Inhibition and reconstitution of oxygen evolution activity in PS II membranes

Preparation	Activity ($\mu\text{mol O}_2 \cdot$ $\text{h}^{-1} \cdot \text{mg Chl}^{-1}$)	Activity (% control)
Control PS II	690	100
Control PS II treated with EGTA/A23187	670	97
2 M NaCl-extracted PS II	240	35
Salt-extracted PS II reconstituted with 17 and 23 kDa polypeptides	630	91
Reconstituted PS II treated with EGTA/A23187	620	90

The procedure for reconstitution of high-salt treated PS II membranes is described in fig. 1

Table 2

Reconstitution of high-salt treated PS II membranes by selective addition of water-soluble polypeptides and Ca^{2+}

Additions to salt extracted PS II membranes (predialyzed against 5 mM EGTA)	Additions to dialysis medium (0.4 M sucrose, 15 mM NaCl, 50 mM Mes, pH 6.0) (2 h dialysis steps)				Polypeptides bound (17 + 23 kDa)	Activity ($\mu\text{mol O}_2 \cdot \text{h}^{-1} \cdot \text{mg Chl}^{-1}$)
	1	2	3	4		
17 and 23 kDa polypeptides in 2 M NaCl (predialyzed against 5 mM EGTA)	5mMEGTA	-	-	-	+	280 (41%) ^a
	5mMEGTA	-	5 mM CaCl_2	-	+	608 (88%)
0.4 M sucrose, 2 M NaCl, 5 mM EGTA, 50 mM Mes (pH 6)	5mMEGTA	-	-	-	-	240 (35%)
	5mMEGTA	-	5 mM CaCl_2	-	-	250 (36%)

^a Percent of untreated control membranes ($690 \mu\text{mol O}_2 \cdot \text{h}^{-1} \cdot \text{mg Chl}^{-1}$)

Loosely-bound Ca^{2+} was removed from the PS II complex and polypeptides during exposure to 2 M NaCl by dialysis of intact PS II membranes (in 2 M NaCl, 1.5 mg Chl/ml) against 5 mM EGTA/2 M NaCl/0.4 M sucrose/50 mM Mes (pH 6.0, 100 vols) for 2 h. The suspension was then centrifuged ($40000 \times g$, 30 min) and the membranes were divided and resuspended in either the high-salt extract containing the 17 and 23 kDa polypeptides or in a solution containing 2 M NaCl/0.4 M sucrose/5 mM EGTA/50 mM Mes (pH 6.0, 1–1.5 mg Chl/ml final conc.). These samples were then dialyzed for 2-h periods (4°C , 1 vol. PS II preparation/100 vols of dialysis medium) as shown. After the final dialysis step the PS II membranes were recovered by centrifugation, washed once with a buffer containing 0.4 M sucrose, 15 mM NaCl, 50 mM Mes (pH 6.0), and finally resuspended in the same buffer

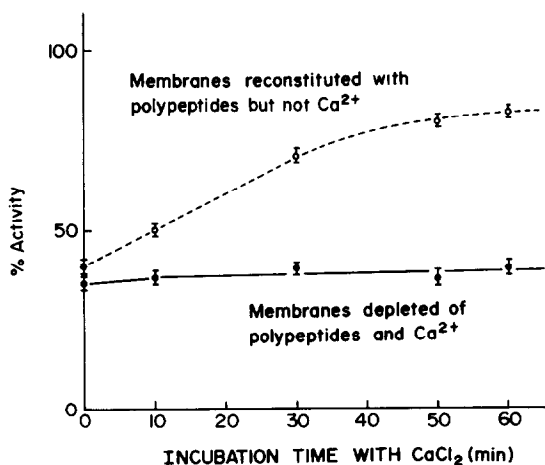


Fig.2. Effect of Ca^{2+} on reactivation of oxygen evolution activity in high-salt/EGTA treated membranes, before (—) and after (---) rebinding of the 17 and 23 kDa polypeptides. PS II membranes were incubated with Ca^{2+} (4°C , 1 mg Chl/10 mM CaCl_2) and after the incubation times shown were diluted into the assay medium (the final Ca^{2+} concentration was $150 \mu\text{M}$). Control (100%) activity: $690 \mu\text{mol O}_2/\text{h}^{-1} \cdot \text{mg Chl}^{-1}$.

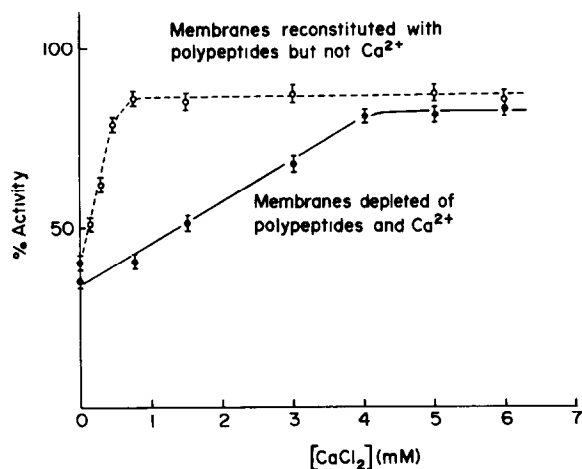


Fig.3. Rates of oxygen evolution as a function of added CaCl_2 . High-salt/EGTA treated PS II membranes before (—) and after (---) rebinding of the 17 and 23 kDa polypeptides were incubated for 10 min (25°C) in the assay medium containing the indicated concentration of CaCl_2 , and then assayed for oxygen evolution activity without any further dilution ($6 \mu\text{g Chl/ml}$). Control (100%) activity: $690 \mu\text{mol O}_2/\text{h}^{-1} \cdot \text{mg Chl}^{-1}$.

the preparations were diluted into assay medium (the final Ca^{2+} concentration was $150 \mu\text{M}$). As can be seen in fig.2, the presence of the rebound 17 and 23 kDa polypeptides appears to enable the reconstituted complex to retain Ca^{2+} as evidenced by a reconstitution of oxygen evolution activity (up to 90% of the unextracted control) not observed with the membranes lacking the two water-soluble polypeptides. If, in fact, the presence of the two polypeptides on the reconstituted PS II membranes has created a high affinity binding site for Ca^{2+} , then this should be reflected in an enhanced Ca^{2+} binding by the reconstituted complex. The data of fig.3 show that this is the case; incubation of membranes at the indicated concentrations of Ca^{2+} followed by assay (without dilution of the Ca^{2+}) demonstrates that when the PS II complex is reconstituted with the 17 and 23 kDa polypeptides the ability of the complex to bind Ca^{2+} is substantially enhanced.

4. DISCUSSION

It is clear from the data presented here that photosynthetically active Ca^{2+} is very tightly bound to the PS II complex. Neither EGTA alone nor in concert with an ionophore (A23187) inactivates the native PS II complex; EGTA is, on the other hand, a potent inhibitor of reconstitution of activity in the dialysis procedure reported here, even though it does not prevent binding of the 23 and 17 kDa polypeptides to sites where subsequent Ca^{2+} addition can reconstitute activity (fig.2,3). We would thus conclude that Ca^{2+} is not required for productive binding of the 17 and 23 kDa polypeptides to PS II membranes, but binding of the 17 and 23 kDa polypeptides to PS II membranes is necessary for tight binding of Ca^{2+} and stimulation of oxygen evolution activity to occur.

Our experiments show clearly that there is a Ca^{2+} -binding site at the oxidizing side of PS II, but do not define the mechanism by which Ca^{2+} promotes oxygen evolution activity. Since Ca^{2+} -binding proteins are very common in biological systems and Ca^{2+} binding is known to induce a conformational response of the receptor protein it is possible that the role of Ca^{2+} in oxygen evolution is structural [14]. Such a structural role could be either a specific involvement of Ca^{2+} in maintaining an active conformation of the manganese

binding site (such a situation is observed in the copper-zinc superoxide dismutases [15]), or a more general effect on the entire structure of the oxidizing side of PS II which could result in a more stable system. Even though a structural role for calcium seems attractive at this point, we cannot rule out a more direct involvement of Ca^{2+} in the photosynthetic water splitting mechanism. We are now exploring further the Ca^{2+} requirement for oxygen evolution activity and carrying out experiments to determine whether the 17 kDa polypeptide is a necessary constituent of the reconstituted oxygen evolving complex.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Science Foundation (PCM 82-18240) to C.F.Y. and USDA/CRGO (5901-0410-9-0344) to G.T.B.

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