Reconstitution of photosynthetic water-splitting activity by the addition of 33 kDa polypeptide to urea-treated PS II reaction center complex

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Treatment with 3 M urea of the oxygen-evolving PS II core complex previously described [(1985) FEBS Lett. 179, 60–64] resulted in approx. 80% release of 33 kDa polypeptide accompanied by partial (40%) inactivation of the water-splitting activity. On the other hand, the treatment only slightly decreased the amount of bound Mn from 2.7 to 2.0 atoms per 50 chlorophyll *a*. Readdition of purified 33 kDa polypeptide to the urea-treated sample almost completely restored the oxygen-evolving activity. These results suggest that the Mn atoms functional for oxygen-evolving activity are located in the PS II reaction center core complex, and that the 33 kDa polypeptide is not essential for water oxidation but is necessary for the full oxygen-evolving activity.

Photosystem II *Reaction center* *Reconstitution* *Urea treatment* *Manganese binding* *33 kDa polypeptide* *Oxygen evolution*

1. INTRODUCTION

Despite numerous works having indicated the possible involvement of 3 polypeptides of 18, 24 and 33 kDa in photosynthetic oxygen evolution [1–6,11–13], there is as yet no conclusive evidence indicating the presence of water-splitting complex composed of these 3 polypeptides. Recent reports rather suggested that ions such as Ca^{2+} or Cl^- can functionally replace the role of either one or all of these 3 polypeptides [7–10].

We have recently succeeded in preparing the PS II core complex capable of oxygen evolution, which contains only a single polypeptide component of 33 kDa, in addition to those of the reaction center core complex, i.e. 47, 43, 32, 30 and 9 kDa [2]. Similar preparations were also reported for *Synechococcus* sp. [14] and spinach using treatment with octyl-β-glucoside [15]. These results strongly suggest that the principal site of water oxidation is associated with the PS II core complex rather than with water-soluble peripheral complex consisting of 18, 24 and 33 kDa polypeptides.

On the other hand, it is well established that Tris, alkaline, or urea treatment releases the 33 kDa polypeptide and Mn and causes inactivation of oxygen evolution in PS II membrane preparations [4,11–13]. In these cases, however, it is usually difficult to restore the oxygen-evolving activity by reading the isolated 33 kDa polypeptide to the extracted membrane. In contrast, the inactivation due to treatment with $CaCl_2$ at higher concentration, which is also known to release the 33 kDa polypeptide, but leaves Mn still associated with the membrane, was partly reversible and restored by the addition of 33 kDa polypeptide or $CaCl_2$ [9].

Here, it is shown that urea treatment of PS II core complex efficiently liberates the 33 kDa

Abbreviations: PS II, photosystem II; Chl, chlorophyll; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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polypeptide and partly inactivates its oxygen evolution, while most of the Mn atoms remain on the complex for a short time. The oxygen evolution of this complex was almost completely restored by the addition of the 33 kDa polypeptide.

2. MATERIALS AND METHODS

The oxygen-evolving PS II core complexes were prepared from spinach as described in [16]. Urea treatment of the purified oxygen-evolving PS II core complexes was carried out as in [4] with minor modifications; the PS II complexes were made to a Chl concentration of 1 mg/ml in 50 mM $CaCl_2$ and 50 mM Mes-NaOH (pH 6.0). An equal volume of 6 M urea was added to the solution to give final concentrations of 25 mM $CaCl_2$, 25 mM Mes-NaOH, 3 M urea and 500 μg Chl/ml. After incubation for 80 min at 0°C in darkness, the sample was subjected to gel-permeation chromatography to eliminate urea using a DEAE-Toyopearl-650 column equilibrated with 50 mM Mes-NaOH (pH 6.0) buffer containing 50 mM $CaCl_2$, and then concentrated by ion-exchange chromatography.

The oxygen-evolving PS II membrane preparations obtained from spinach were washed with 2 M NaCl to remove the 18 and 24 kDa polypeptides as in [3]. The particles were then treated with 1 M $CaCl_2$ and centrifuged (35000 × g, 15 min) to release the 33 kDa polypeptide as in [9]. The resulting supernatant containing the 33 kDa polypeptide was dialyzed against 50 mM Mes-NaOH (pH 6.0) buffer overnight followed by concentration in a micro-ultrafiltration system (model 8 MC, Amicon, MA, USA). The urea-treated PS II core complexes were diluted to 200 μg/ml with 50 mM Mes-NaOH (pH 6.0) and 50 mM $CaCl_2$, and then subjected to reconstitution by the addition of isolated 33 kDa polypeptide in an amount at a nearly equal ratio with the 43–47 kDa polypeptide as in PS II particles. After incubation in darkness at 0°C for 1 h, the sample was subjected to photochemical measurements and further biochemical analysis.

Oxygen evolution was measured at 20°C with a Clark-type oxygen electrode (model 53, Yellow Springs Instruments, USA) in a medium containing 400 mM sucrose, 30 mM $CaCl_2$, 600 μM phenyl-p-benzoquinone, 2 mM ferricyanide and 50 mM Mes-NaOH (pH 6.0) as in [16]. Polypeptides were analyzed by SDS-PAGE in the presence of 6 M urea in the analyzing gel using a 14% polyacrylamide gradient as described in [18]. The relative amounts of the 33 kDa polypeptide were estimated according to the peak areas of the stained bands on the densitogram. The Mn content in the sample was determined using an inductively coupled plasma emission spectrometer (Spectra Span V dual source multi-element system, Bechman, Berkeley, USA).

3. RESULTS AND DISCUSSION

Fig.1 shows the activity of photochemical oxygen evolution supported by phenyl-p-benzoquinone plus ferricyanide for untreated, urea-treated and reconstituted PS II core complex. The purified oxygen-evolving PS II core complex used in this experiment exhibited a typical rate of oxygen evolution of 239 μmol O_2 mg·Chl^{-1}·h^{-1}. Urea treatment significantly decreased the activity to 146 μmol O_2 mg·Chl^{-1}·h^{-1} in this experiment. The oxygen evolution was almost completely restored by the addition of excess amounts of 33 kDa polypeptide. The rates of oxygen evolution before and after reconstitution were estimated to be 61 and 94%, respectively, of the original activity of the untreated complex.

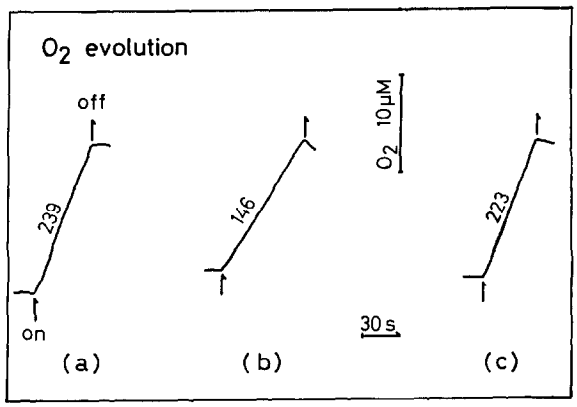

Fig.1. Oxygen evolution by urea-treated and reconstituted PS II core complex; 6 μg Chl/ml. (a) Untreated oxygen-evolving PS II reaction center complex, (b) urea-treated complex, (c) reconstituted complex. Values beside each progress curve indicate the rate of oxygen evolution in μmol O_2·mg Chl^{-1}·h^{-1}. See text for further explanation.

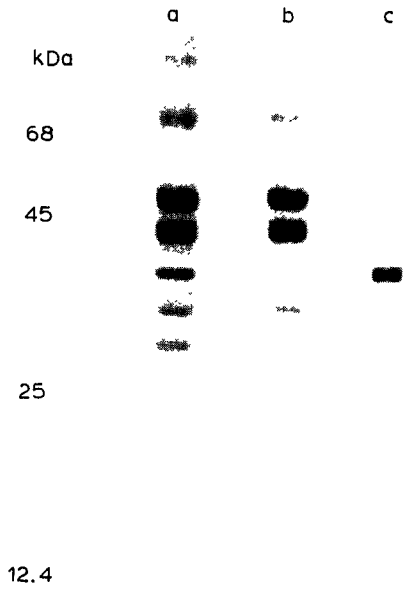

Fig.2. SDS-PAGE profile of oxygen-evolving PS II core complex: (a) Untreated oxygen-evolving PS II reaction center complex, (b) urea-treated complex, (c) 33 kDa polypeptide prepared as in section 2. Positions of marker proteins shown on the left: bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.4 kDa).

Fig.2 shows the polypeptide profile of the samples before and after these treatments. The untreated oxygen-evolving PS II core complex contains 33 kDa polypeptide, in addition to those of 5 reaction center polypeptides, i.e. 47, 43, 32, 30 and 9 kDa [17]. Urea treatment resulted in about 80% of the 33 kDa polypeptide present in the original complex being removed, whereas the oxygen-evolving activity decreased only about 40% (table 1). Thus, the activity is not proportional to the amount of bound 33 kDa polypeptide as far as these two samples are concerned. In some experiments a relatively higher rate of oxygen evolution was observed even in the presence of a minute amount of 33 kDa polypeptide in the complex.

The abundance of Mn in the PS II core complex capable of oxygen evolution before and after urea treatment is listed in table 1. In contrast with the PS II complex inactive in oxygen evolution prepared at pH 7.2 [19], which contains less than 1 Mn atom per reaction center, the oxygen-evolving complex reported here retains about 3 Mn atoms per 50 Chl. A relatively small portion (about 25%) of Mn was extracted from the complex after urea treatment along with liberation of 80% of the 33 kDa polypeptide and 40% inactivation in the oxygen-evolving activity.

Table 1

Mn abundance, amount of 33 kDa protein bound and oxygen-evolving activity in urea-treated and reconstituted oxygen-evolving PS II core complex

Type of PS II complex	O_2 evolution[a] ($\mu mol \cdot mg^{-1}$ Chl $\cdot h^{-1}$)	33 kDa protein bound (%)	Mn abundance (atoms/50 Chl)
Untreated O_2-evolving PS II core complex	239 (100%)	100	2.7
Urea-treated O_2-evolving PS II core complex	146 (61%)	20	2.0
Reconstituted O_2-evolving PS II core complex	223 (94%)	97	–
PS II core complex prepared at pH 7.2[b]	0	0	0.7

[a] Data from fig.1
[b] Data from [17]

Our results strongly suggest that the Mn atoms responsible for oxygen-evolving activity are located on the PS II core complex consisting of 5 polypeptides of the reaction center, and thus the core complex is the principal site for the catalysis of water oxidation. The 33 kDa polypeptide may not be absolutely essential for the activity of water oxidation, but it is evidently necessary for manifestation of the full activity. The 33 kDa polypeptide may be essential as a shield for Mn atoms functional for oxygen evolution as postulated for membrane preparations [9,20].

We have attempted to liberate the 33 kDa polypeptide of the core complex by treatment at pH 7.2 or with concentrated $CaCl_2$. Treatment of the core complex with Tris buffer at pH 7.2 for 100 min at 0°C released practically all of the 33 kDa polypeptide and the oxygen-evolving activity was completely diminished. Readdition of the isolated 33 kDa polypeptide to the inactivated complex, however, was unsuccessful in reconstituting its oxygen evolution, probably due to the loss of Mn upon pH treatment. In the case of $CaCl_2$ extraction, the 33 kDa polypeptide could not be liberated even with 1 M $CaCl_2$ at 0°C.

In [16], we have described the presence of 22 kDa polypeptide in the oxygen-evolving PS II core complex. However, this is not reproducible and should thus be regarded as a contamination.

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