The oxygen-evolving photosystem II core complex*

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Digitonin extracts of spinach thylakoid membranes obtained as 100000 × g supernatant have been shown to retain part of the oxygen-evolving activity if the extraction is carried out at acidic pH. The photosystem II reaction center complex with a uniform particle size of 300–500 kDa was purified from the extracts by ion-exchange/gel-permeation chromatography performed at pH 6.0 using DEAE-Toyopearl-650. The purified materials contained only two polypeptides of about 22 (not 24) and 33 kDa, in addition to those of the reaction center core complex, i.e., 47, 43, 30 and 10 kDa. The purified complex was fairly active in the photochemical oxygen evolution supported by phenyl-*p*-benzoquinone (~150 μmol oxygen evolved · mg $chl^{-1} \cdot h^{-}$) and DCIP photoreduction with water. From these results it is concluded that the principal site of water oxidation is associated rather with the photosystem II core complex than with the water soluble peripheral complex consisting of 18, 24 and 33 kDa polypeptides and that the thylakoid membrane structure is not essential for manifestation of oxygen-evolving activity.

Photosystem II *Reaction center* *O_2 evolution* *Digitonin extraction* *Polypeptide composition* *Photosynthesis*

1. INTRODUCTION

The possible involvement in photosynthetic oxygen evolution, of the 3 polypeptides in the hydrophilic domain of the thylakoid membrane with molecular masses of 18, 24 and 33 kDa, has been well-documented [1–11,18,19]. However, there is as yet no direct evidence indicating the presence of the hypothetical complex 'water dehydrogenase', as a definite chemical entity distinct from the PS II reaction center complex. A lot of work has been done either on membrane preparations with associated PS II reaction centers [2–5] or on isolated polypeptides [7,9–11]. However, the binding site of Mn or the presence of other catalytic component(s) on the isolated polypeptides has not been successfully determined (however, see [8]).

In contrast, recent reports indicate that ions such as Ca^{2+} or Cl^{-} can support the oxygen-evolving activity in membrane preparations depleted of either one or all of the 3 polypeptides [7,9–11]. In view of the above observations together with the fact that there is a very rapid and efficient electron transport from water to the PS II reaction center [12], it is tempting to assume that the catalytic site for oxygen evolution is on the polypeptide of the reaction center core complex [13] rather than on the water-soluble peripheral proteins.

Here we report a new preparation of the PS II core complex capable of oxygen evolution. The analysis of this material provides evidence supporting the PS II core complex as the principal site for the catalysis of water oxidation.

* This paper is dedicated to Professor Warren L. Butler

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

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2. MATERIALS AND METHODS

NaBr-washed thylakoid membranes and digitonin extracts were prepared from spinach as in [14] with some modifications: 50 mM Mes-NaOH (pH 6.0) was used instead of 50 mM Tris–HCl (pH 7.2) used in the original procedure, for washing and extracting thylakoid membranes; NaCl was omitted from the extraction medium (see section 3). The PS II reaction center complex was purified from digitonin extracts by a simple two-step chromatographic method using DEAE-Toyopearl (type 650S, Toyo Soda, Tokyo) as described (see section 3 and [15]).

The rate of photoreduction of DCIP, either with or without the added artificial electron donor, 1,5-diphenylcarbazide, was measured photometrically (ΔA_{580}, 12.9 $mM^{-1} \cdot cm^{-1}$) at 25°C in a medium containing 130 mM NaCl, 30 μM DCIP, 50 mM $CaCl_2$ and 50 mM Mes-NaOH (pH 6.0). The concentration of 1,5-diphenylcarbazide was 150 μM and that of the PS II preparations was equivalent to 10 μg chl $\cdot$ ml^{-1}. Actinic light was provided by an incandescent lamp combined with heat-absorbing filters (Hoya, HA-50) and a red cut-off filter (Toshiba, V-R65). Oxygen evolution was measured at 25°C with a Clark-type oxygen electrode (model 53, Yellow Springs Instruments, USA) in a medium containing 400 mM sucrose, 30 mM NaCl, 25 mM $CaCl_2$, 600 μM phenyl-*p*-benzoquinone and 50 mM Mes-NaOH (pH 6.5). The concentration of PS II reaction center preparations was equivalent to 20 μg chl $\cdot$ ml^{-1}. White actinic light was provided by an incandescent lamp combined with a 10 cm layer of $CuSO_4$ filter and a blue cut-off filter (Toshiba, V-Y 39). The light intensity used was to the saturation point for oxygen evolution. The polypeptide profiles of the particles were analyzed by SDS–PAGE carried out as in [16] with modifications: 4 M urea was included in the analyzing gel and a 10–20% polyacrylamide gradient was made. The particle size of the complex was estimated chromatographically using a gel-permeation column (type SW3000G, Toyo Soda) as in [17].

3. RESULTS AND DISCUSSION

The previously described highly purified PS II core complex [13,14] was inactive in oxygen evolution. The purification step destroying the oxygen-evolving activity has been shown to be that of digitonin extraction of thylakoid membranes [15]. Here, we tried to improve the solubilization process by changing the pH of the extracting buffer solution, so that the oxygen-evolving capacity is not inactivated. As shown in fig.1, the photochemical activity of DCIP photoreduction with water of the small particles not sedimenting at 100000 × *g* for 1 h markedly depended on the pH of the extracting medium and was relatively stable at acidic pH where oxygen-evolving activity was shown to be optimal [18]. On the other hand, the same activity with added diphenylcarbazide (fig.1) as well as the extent of solubilization (not shown) were practically independent of pH within the experimental conditions tested. The specific activity of DCIP photoreduction with water of this preparation was only one-third of the same activity with an artificial electron donor, indicating that digitonin extraction is still deleterious to the oxygen-evolving activity even at optimal conditions for the extraction (pH 6.0).

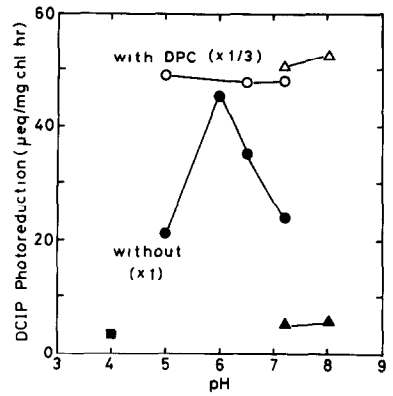

Fig.1. Digitonin extraction of PS II particles from spinach thylakoids at different pH. Spinach thylakoid membranes were treated with 1.0% digitonin in 50 mM Tris-HCl (triangles), Mes-NaOH (circles) or potassium-phthalate-NaOH (square) buffer at 2.0 mg chl $\cdot$ ml^{-1} for 1 h at 0°C, and then the suspension was centrifuged at 100000 × *g* for 1 h at 4°C. The photochemical activity of DCIP photoreduction either without (filled symbols) or with 1,5-diphenylcarbazide (open symbols) was measured as described in section 2. Note that the activity with diphenylcarbazide is shown in reduced scale (one-third).

We made use of this extract capable of oxygen evolution obtained at pH 6.0 for further purification of the PS II core complex by applying a newly developed, two-step, chromatographic method [15]. Because the oxygen-evolving activity in the extract was relatively large at pH 6.0, we used Mes-NaOH (pH 6.0) instead of Tris-HCl (pH 7.2) in the following chromatographic separations. We also modified the original procedure [15] to some extent regarding the ionic strength of the elution buffer. Briefly, the above-mentioned digitonin extract was adsorbed onto a DEAE-Toyopearl column and eluted by an NaCl gradient (50–170 mM) in 50 mM Mes-NaOH (pH 6.0). Then the PS II-enriched fractions in the eluent were applied to another DEAE-Toyopearl column equilibrated with the same buffer containing 150 mM NaCl and further purified by gel-filtration/ion-exchange chromatography [15].

The purified PS II particles prepared by this procedure were practically free from light-harvesting chlorophyll *a/b*-protein and the PS I reaction center complex as shown by their absorption spectrum (fig.2), polypeptide profiles (fig.4) and photochemical activities. The absorption spectrum of the purified preparation shown in fig.2 exhibits typical characteristics of the PS II reaction center complex reported in [13], including the red absorption maximum at 674 nm, the absence of a noticeable shoulder at 650 nm due to chlorophyll *b* absorption and the presence of a small peak around 540 nm often ascribed to pheophytin absorption. High-performance liquid chromatography of the purified material using a gel permeation column as in [17] indicated the presence of a single component of 300–500 kDa as monitored at 280 and 674 nm. The estimated size is similar to the reported value for the PS II reaction center extracted and purified at pH 7.2 [17]. This experiment suggests that the particle is no longer in membrane fragments, but in the state of a uniform chlorophyll–protein complex. Lipid analysis also supported this interpretation.

The purified chlorophyll–protein complex is highly active in PS II photochemistry and significantly active in oxygen evolution as shown by DCIP photoreduction with water as the electron donor (fig.3A) and the photochemical oxygen evolution supported by phenyl-*p*-benzoquinone (fig.3B). However, the rate of DCIP photoreduction with diphenylcarbazide is about 3-times higher than that without an added artificial electron donor, even in purified materials. This is inconsistent with the data in fig.1. The oxygen-evolving activity of the preparations after digitonin extraction was very sensitive to actinic light illumination (photoinhibition) as reported for thylakoid membranes [17], but was protected by the presence of $CaCl_2$ in the reaction mixture.

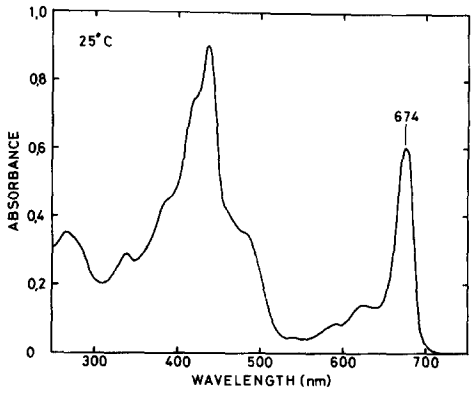

Fig.2. Absorption spectrum at room temperature of the purified oxygen-evolving PS II core complex.

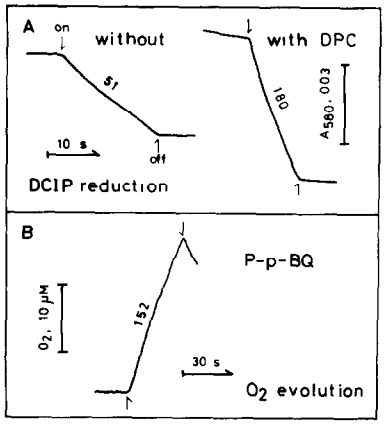

Fig.3. Photochemical activities of the purified oxygen-evolving PS II core complex. Photoreduction of DCIP (A) in the absence or presence of 1,5-diphenylcarbazide, and photochemical oxygen evolution supported by phenyl-*p*-benzoquinone (B) were measured as described in section 2. The number beside each progress curve indicates the rate of reaction in μmol DCIP (A) or oxygen (B)·mg^{-1} chl·h^{-1}.

Fig.4 shows the polypeptide profile of the purified oxygen-evolving PS II core complex. The profile exhibits polypeptides of the PS II reaction center described for the materials prepared at pH 7.2, i.e., two chlorophyll binding polypeptides of 43 and 47 kDa, a diffuse composite band of about 30 kDa observed at 4 M urea used in this experiment, including herbicide binding polypeptide, and a small polypeptide of about 10 kDa for cytochrome *b*-559. Only two additional polypeptides are evident on the profile. One of these polypeptides is a sharp band probably corresponding to the 33 kDa polypeptide of the hypothetical oxygen-evolving complex [3]. One may expect an essential role for this polypeptide in oxygen evolution, however, the function of this polypeptide in oxygen evolution is not firmly established. It is clear that even without this polypeptide the oxygen-evolving activity of the membrane fragments has been shown to be partly active in some experiments [9,10]. On the other hand, the other two components of the oxygen-evolving complex, i.e., 18 and 24 kDa, are missing in the oxygen-evolving PS II reaction center complex reported here. This is in agreement with opinions that these two polypeptides are not obligatory for the oxygen-evolving reaction [7,9,19]. The purified complex contains small but substantial amounts of a polypeptide of about 22 kDa. This polypeptide is usually observed in reported profiles of 'highly active oxygen evolving membrane fragments' [3,4,7, 11], but the function of this polypeptide in oxygen evolution has not been analyzed.

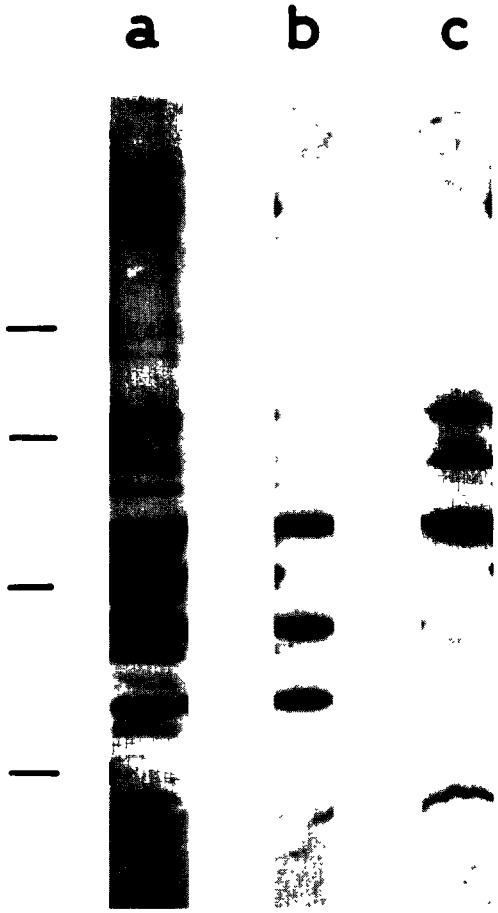

Fig.4. SDS–PAGE profile of the purified oxygen-evolving PS II core complex: (a) NaBr-treated chloroplasts; (b) 1 M $CaCl_2$-extracts of PS II particles consisted of 18, 24 and 33 kDa polypeptides prepared as in [4]; (c) purified PS II core complex. Bars beside indicate the positions of marker proteins; bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.4 kDa), respectively, from top to bottom.

4. CONCLUSIONS

From the data presented here, the following conclusions can be derived.

(i) It is unlikely that there is an enzyme complex distinct from the PS II core complex and responsible for photosynthetic water oxidation.

(ii) The presence of the thylakoid membrane structure and, at least, the two polypeptides of 18 and 24 kDa are not essential for manifestation of photosynthetic oxygen evolution.

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