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# Isolation and characterization of Photosystem II complexes which lack light-harvesting chlorophyll *a/b* proteins but retain three extrinsic proteins related to oxygen evolution from spinach

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**Oxygen-evolving Photosystem II (PS II) complexes, which were largely deprived of major light-harvesting chlorophyll *a/b* proteins (LHC II) but still associated with the 33 kDa, 23 kDa and 17 kDa extrinsic proteins related to oxygen evolution, were isolated from spinach oxygen-evolving PS II membranes with a non-ionic detergent, *n*-heptyl thioglucoside. A minor antenna chlorophyll-protein (CP 29) was present but in reducted amounts. The complexes contained all the constituent subunits of PS II reaction center core complexes, the 47 kDa and 43 kDa chlorophyll-carrying proteins, the D1 and D2 proteins and cytochrome *b*-559. In addition, three hydrophobic proteins of 29 kDa (CP 29 apoprotein), 20 kDa and 10 kDa were present. The antenna size was 80 chlorophyll *a* per $Q_A$, or 76 chlorophyll *a* per 4 Mn, and the complexes contained about 1 $Ca^{2+}$ per PS II. With phenyl- or dichloro-*p*-benzoquinone as electron acceptor, the complexes showed high rates of oxygen evolution in the absence of exogenously added $Ca^{2+}$. The activity became, however, strongly $Ca^{2+}$-dependent when the 23 kDa and 17 kDa proteins, but not the bound $Ca^{2+}$, had been removed with 1.5 M NaCl. The $Ca^{2+}$ requirement disappeared on reconstitution of the complexes with the two proteins. The complexes were compared with other oxygen-evolving preparations having different polypeptide compositions and functions of several subunit proteins and $Ca^{2+}$ in PS II electron transport are discussed.**

## Introduction

Photosynthetic oxygen evolution takes place in a discrete multiprotein complex of the thylakoid membranes which carries chlorophyll *a*, carotenoids and various reaction components. Oxygen-evolving PS II preparations isolated from the thylakoid membranes of higher plants with Triton X-100 have been widely used for investigation of the functions of the three extrinsic proteins with molecular masses of about 33, 23 and 17 kDa and cofactors such as chloride and calcium in water oxidation [1–8]. However, there are still membrane fragments containing a large amount of LHC II, together with many other proteins of unknown function. In order to determine the molecular organization of the oxygen-evolving system, efforts have been exerted to isolate oxygen-evolving preparations with simpler protein compositions [9,10]. PS II complexes have been purified from *Synechococcus* which evolve oxygen at rates exceeding 1000 μmol/mg Chl per h [11]. Abundances of reaction components of the cyanobacterial complexes have been determined and a composition of a minimum unit for oxygen evolution has been proposed [11]. PS II complexes purified by Ikeuchi et al. [12,13] from spinach and wheat with β-octyl glucoside are also highly active in oxygen evolution. The complexes which lack the 23 and 17 kDa extrinsic proteins are, however, not competent by themselves and high rates of oxygen evolution are attained only in the presence of a nonphysiologically high concentration of $Ca^{2+}$. Successful reconstitution of the purified complexes with the 23 and 17 kDa proteins has not yet been demonstrated and involvement of hydrophobic 10 and 22 kDa proteins in the binding of the extrinsic 23 kDa protein has been suggested [14]. For determination of the func-

Abbreviations: PS II, Photosystem II; LHC II, light-harvesting chlorophyll *a/b* protein of PS II; Mes, 4-morpholineethanesulfonic acid; $Q_A$ and $Q_B$, primary and secondary quinone acceptors of PS II, respectively; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; PBQ, phenyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone.

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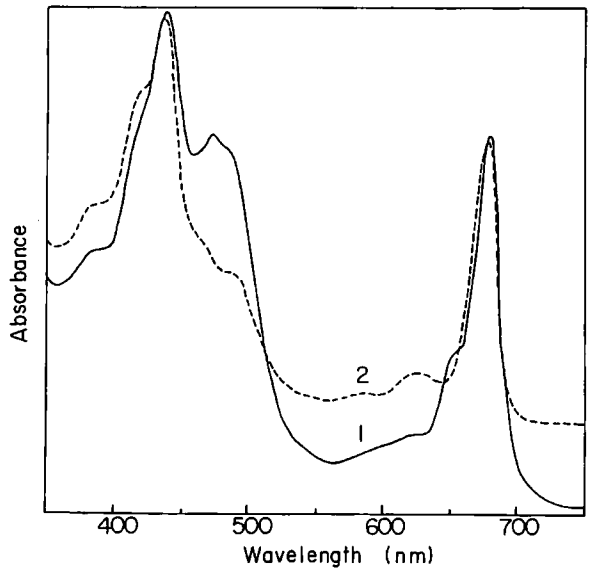


Fig. 1. Absorption spectra of Triton-PS II membranes (1) and oxygen-evolving complexes (2).

glucoside [20]. The terminology of Camm and Green was used for chlorophyll proteins [20]. CP 27 is a monomer of LHC, and CP 64, a predominant chlorophyll band resolved from the membrane preparations, is a trimer of CP 27. CP 47 and CP 43 are intrinsic chlorophyll-carrying proteins of the PS II reaction center complexes and CP 29 is an antenna chlorophyll protein of PS II. Note that the two LHC II species, CP 64 and CP 27, were not resolved from the complexes. The amount of CP 29 relative to that of CP 47 or CP 43 was also considerably reduced in the complexes.

Polypeptide compositions of the complexes were examined by SDS-gel electrophoresis under denaturing conditions (Fig. 3). Dense bands resolved from the PS

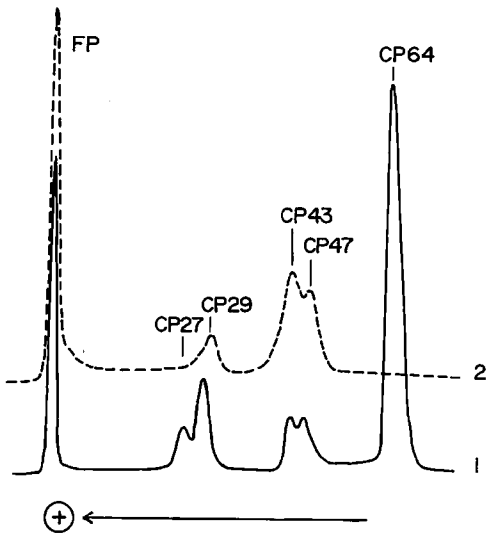


Fig. 2. Densitometer tracings of chlorophyll-protein complexes resolved by SDS-gel electrophoresis. (1) Triton-PS II membranes; (2) oxygen-evolving complexes.

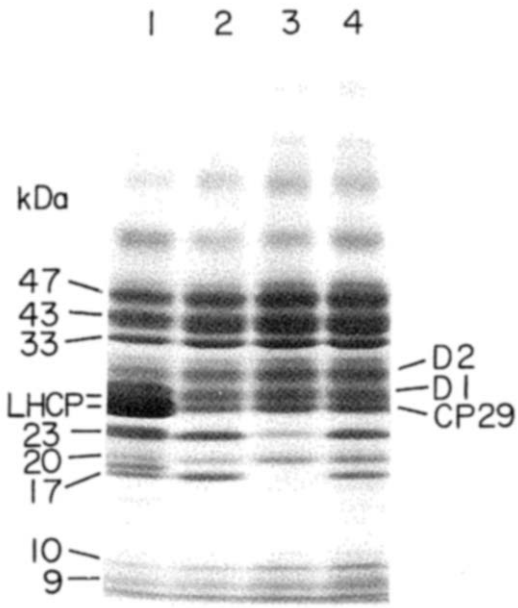


Fig. 3. Polypeptide compositions of Triton-PS II membranes (lane 1), oxygen-evolving complexes (lane 2), NaCl-washed complexes (lane 3) and reconstituted complexes (lane 4). NaCl-treatment was carried out under room light. Other procedures for NaCl wash and reconstitution were described in Materials and Methods.

II membranes in the molecular mass region 27–30 kDa are apoproteins of LHC II (lane 1). The bands were mostly absent from the complexes and a band appeared at 29 kDa is ascribed to the apoprotein of CP 29 [20] (lane 2). The complexes contained all the subunit proteins of PS II reaction center core complexes; the 47 and 43 kDa chlorophyll-carrying proteins, the D1 and D2 proteins and the 9 kDa cytochrome *b*-559 apoprotein. In addition, there were two proteins of 20 and 10 kDa. They correspond to the 22 and 10 kDa proteins which have previously been isolated and charaterized from spinach PS II membranes [14,26]. The complexes were found to retain the three extrinsic proteins of 33, 23 and 17 kDa which are involved in oxygen evolution [27]. When compared with the original PS II membranes with the 47 kDa protein as a reference, losses of the 23 and 17 kDa proteins during *n*-heptyl thioglucoside treatment were only 10%, and essentially all the 33 kDa protein remained bound to the complexes.

The complexes evolved oxygen at high rates with phenyl-*p*-benzoquinone as electron acceptor (Table I). Dichloro-*p*-benzoquinone also supported a substantial rate of oxygen evolution, whereas ferricyanide was a poor electron acceptor. Note that the addition of 5 mM $Ca^{2+}$ enhanced the oxygen-evolving activity only by 12–18%. Such a minor $Ca^{2+}$ effect has occasionally been observed in Triton-PS II membranes. The activity was not appreciably affected by the addition of 2 mM EDTA (data not shown), indicating that oxygen evolution is not supported by loosely bound $Ca^{2+}$. The oxygen-evolving activity of the complexes was strongly

TABLE I

*Oxygen evolution with different electron acceptors*

Oxygen evolution was measured in a medium containing 40 mM Mes-NaOH (pH 6.0), 10 mM NaCl, 5 mM $MgCl_2$ and 0.4 M sucrose in the absence or presence of 5 mM $CaCl_2$. Where indicated, 10 μM DCMU added.

| Preparations | Electron acceptors | Oxygen evolution (μmol $O_2$/mg Chl per h) | | |
|---|---|---|---|---|
| | | −Ca | +Ca | |
| | | −DCMU | −DCMU | +DCMU |
| Triton-PS II membranes | 0.4 mM PBQ | 540 | 639 | 0 |
| | 0.4 mM DCBQ | 409 | 475 | 10 |
| | 1.0 mM $Fe(CN)_6^{-3}$ | 115 | 115 | 16 |
| Complexes | 0.4 mM PBQ | 945 | 1114 | 22 |
| | 0.4 mM DCBQ | 713 | 799 | 50 |
| | 1.0 mM $Fe(CN)_6^{-3}$ | 315 | 371 | 37 |

inhibited by 10 μM DCMU. The complexes did not require a detergent such as digitonin to show a high rate of oxygen evolution. The data suggest that the complexes have relatively intact electron transport involving a functional $Q_B$, the second quinone acceptor of PS II, on the reducing side of PS II.

TABLE II

*Effect of NaCl wash on oxygen evolution of the complexes*

Procedures for NaCl wash and reconstitution were described in Materials and Methods. Oxygen evolution was determined as in Table I with 0.4 mM phenyl-*p*-benzoquinone as electron acceptor.

| Treatments | Oxygen evolution (μmol $O_2$/mg Chl per h) | |
|---|---|---|
| | −Ca | +5 mM Ca |
| Untreated | 950 | 1110 |
| NaCl-washed | 169 | 540 |
| Reconstituted | 418 | 466 |

TABLE III

*Relative abundances of $Q_A$, Mn and Ca in Triton-PS II membranes and the oxygen-evolving complexes*

| Components | Abundance (mol Chl/mol) | |
|---|---|---|
| | Triton-PS II membranes | complexes |
| $Q_A$ | 225±12 | 80± 5 |
| Mn | 57± 3 | 19± 1 |
| Ca | 107± 5 | 82±16 |

TABLE IV

*Effect of NaCl wash on Ca content of the oxygen-evolving complexes*

The complexes were treated with 1.5 M NaCl in the dark or in the light (1500 lux).

| $Ca/Q_A$ ratio | | |
|---|---|---|
| untreated | NaCl-washed (dark) | NaCl-washed (light) |
| 0.92±0.2 | 1.2±0.2 | 1.1±0.2 |

Treatment of the complexes with 1.5 M NaCl caused a strong inactivation of oxygen evolution (Table II). The 23 and 17 kDa proteins were solubilized by the treatment (see Fig. 3, lane 3). The lost activity was significantly but not completely restored by the addition of 5 mM $Ca^{2+}$. The released 23 and 17 kDa proteins rebound to the complexes when NaCl concentration was reduced by a prolonged dialysis (Fig. 3, lane 4). Oxygen evolution was considerably restored by the reconstitution with the two proteins and $Ca^{2+}$ had only a small effect on the activity of the reconstituted complexes (Table II).

Table III shows abundances of reaction components in the oxygen-evolving complexes. $Q_A$, the first quinone acceptor of PS II, was present at a chlorophyll to $Q_A$ ratio of 80. Thus the complexes have a significantly larger antenna size than highly purified PS II complexes which show the chlorophyll to $Q_A$ ratios of about 50 [11,13,15,17]. The difference is ascribed to the association of CP29 with our complexes. The Mn cluster seems to have been well preserved in the complexes because the antenna size estimated on the basis of 4 Mn was 76. Triton-PS II membranes contained about 2 $Ca^{2+}$ per PS II, in agreement with the previous estimations [15,17,28,29]. In contrast, there was only one $Ca^{2+}$ per $Q_A$ or 4 Mn in the complexes, indicating that *n*-heptyl thioglucoside treatment removed one of the two bound $Ca^{2+}$. Table IV shows that the $Ca^{2+}$ content of the complexes was not appreciably affected by the NaCl wash either under dark or light conditions. Thus the $Ca^{2+}$ requirement created by the NaCl wash cannot be ascribed to release of the bound $Ca^{2+}$.

## Discussion

Properties of the PS II complexes prepared in the present work are summarized in Table V. For the comparison, the data of other PS II preparations highly

active in oxygen evolution are also presented. Two oxygen-evolving complexes purified by Ikeuchi et al. [12,13] from spinach and wheat (5th column) and by Ohno et al. [11] from the thermophilic cyanobacterium *Synechococcus* (the last column), which was recently identified as *Synechococcus elongatus Nägeli* [30], have similar simple protein compositions. They consist of the 47 and 43 kDa chlorophyll-carrying proteins, cytochrome *b*-559 and the extrinsic 33 (35) kDa protein. The complexes prepared in the present work additionally contain CP 29, two hydrophobic proteins of 20 and 10 kDa and two hydrophilic proteins of 23 and 17 kDa (2nd column). PS II preparations isolated from the rice chlorina mutant (3rd column) have similar protein compositions except for CP 29, which is absent from the original mutant membranes [15]. Ghanotakis et al. [31–33] have isolated a PS II preparation called 'PS II reaction center complex' by exposing PS II membranes to β-octyl glucoside in the presence of high ionic strength (4th column). The preparation retains CP 29 and two intrinsic proteins of 22 and 10 kDa but, in contrast to our preparation and rice mutant complexes, lacks the extrinsic 23 and 17 kDa proteins. More purified complexes of Ghanotakis et al. [31–33] are essentially the same as the complexes of Ikeuchi et al. [12,13]. In the following, photochemical activities and abundances of reaction components of PS II preparations with different polypeptide compositions are compared.

Oxygen-evolving rates of PS II complexes are comparable to each other on the basis of chlorophyll. However, the complexes isolated with *n*-heptyl thioglucoside have a larger antenna size than other purified preparations. When compared on the basis of PS II ($Q_A$ or 4 Mn), therefore, the oxygen-evolving activity of the complexes is highest among highly or partially purified PS II complexes. Thus the complexes have more intact oxygen-evolving system than others.

Table V provides important information on the function of the extrinsic 23 kDa protein. NaCl wash of PS II preparations creates a strong $Ca^{2+}$ requirement for oxygen evolution [8,18]. This has been postulated to be caused by release of $Ca^{2+}$; removal of the 23 kDa protein destabilizes $Ca^{2+}$ functioning in PS II electron transport and the metal cations added restore oxygen evolution or related reactions by rebinding to the original functional sites. Various functional sites of $Ca^{2+}$ in oxygen evolution have been proposed based on this postulation [8,18,33–39]. Table V shows, however, that the $Ca^{2+}$ requirement is not related to $Ca^{2+}$ contents of preparations. All the partially or highly purified oxygen-evolving complexes isolated from higher plants contain one bound $Ca^{2+}$ per PS II (except for the complexes prepared in Refs. 31, 33 of which $Ca^{2+}$ content has not yet been reported), which is half that of Triton-PS II membranes. Nevertheless, the $Ca^{2+}$ requirement is observed only the the two complexes which lack the 23 and 17 kDa proteins (4th and 5th columns). Thus, appearance of the $Ca^{2+}$ requirement parallels loss of the extrinsic protein.

The present work further showed that a strong $Ca^{2+}$ requirement is induced by NaCl wash of the *n*-heptyl thioglucoside-complexes, which removed the 23 and 17 kDa proteins but not the bound $Ca^{2+}$. The $Ca^{2+}$-insensitive activity was restored on reconstitution of the complexes with the two proteins. Thus, all the results consistently led to the conclusion that the $Ca^{2+}$ requirement is a consequence of solubilization of the 23 (and 17) kDa protein. The 23 kDa protein would be needed to maintain the conformation of the oxygen-evolving complex optimal for the activity. A high concentration

TABLE V

*Comparison of oxygen-evolving PS II preparations*

| | Spinach PS II membranes (present work and [17]) | Spinach complexes (present work) | Rice mutant complexes [15] | Spinach complexes [31,33] | Wheat complexes [13] | *Synechococcus* complexes [11] |
|---|---|---|---|---|---|---|
| μmol $O_2$/mg Chl per h | 640 | 1100 | 1100 | 940 | 1500 | 1300 |
| $Ca^{2+}$ requirement | low | low | low | high | high | low |
| Chl/PS II | 200–250 | 80 | 53 | 70 | 40 | 50 |
| Ca/PS II | 2.0 | 1.0 | 1.0 | nd | 1.0 | 0.7 |
| Best $e^-$ acceptor | DCBQ | PBQ | PBQ,DCBQ | DCBQ | $Fe(CN)_6^{-3}$ | $Fe(CN)_6^{-3}$ |
| DCMU sensitivity | high | high | high | high | low | high |
| LHC II | bound | – | – | – | – | – |
| CP 29 | bound | bound | – | bound | – | – |
| Associated hydrophobic proteins (kDa) | 22 | 20 | 22 | 22 | – | – |
| | 10 | 10 | 10 | 10 | – | 8 |
| Associated extrinsic proteins (kDa) | 33 | 33 | 33 | 33 | 33 | 35 |
| | 23 | 23 | 21 | – | – | – |
| | 17 | 17 | 15 | – | – | – |

of $Ca^{2+}$ stimulates oxygen evolution in the complexes depleted of the 23 kDa protein by restoring the functional conformation of the complexes [15,17].

Addition of high concentrations of $Ca^{2+}$ is not needed to promote high rates of oxygen evolution in *Synechococcus* complexes which have no proteins corresponding to the 23 and 17 kDa proteins of higher plants [11]. Because the occurrence of the two water-soluble proteins has never been reported in cyanobacteria, the 35 kDa protein (which corresponds to the 33 kDa protein of higher plants) alone would be sufficient to maintain the functional conformation of the cyanobacterial oxygen-evolving complexes, provided that high concentrations of sucrose or glycerol is present [10,11].

The two hydrophobic proteins of 20 (22) and 10 kDa have been suggested to be involved in the association of the extrinsic 23 and 17 kDa proteins [14]. Table V appears to provide a support for this assumption. The two hydrophobic proteins are present in our complexes and rice mutant preparations which are associated with the 23 and 17 kDa proteins and in the complexes of Ghanotakis et al. [40] which were reported to rebind the extrinsic proteins. Only the Ikeuchi's complexes which have lost the two hydrophobic proteins cannot be reconstituted with the 23 and 17 kDa proteins [12–14].

The highly purified complexes of Ikeuchi et al. [12,13] are also different from other preparations in the mode of acceptor reduction. The complexes show high rates of oxygen evolution with ferricyanide but not with phenyl-*p*-benzoquinone as electron acceptor. Ferricyanide-supported oxygen evolution is insensitive to DCMU. In contrast, other preparations from higher plants are highly sensitive to DCMU and show a strong preference of phenyl-*p*-benzoquinone over ferricyanide, suggesting the presence of the functional $Q_B$. The difference may be ascribed to different degrees of detergent effects. Recently, however, the hydrophobic 10 kDa protein, which is missing only in the highly purified complexes, has been suggested to involve in electron transport or reducing side of PS II [41]. Because phosphorylation of the protein suppresses electron transport from $Q_A$ to $Q_B$, it is argued that the protein, in its dephosphorylated state, aids efficient electron transfer from the reaction center to the attendant plastoquinone pool [41]. Thus the altered electron transport of the complexes may as well be related to the loss of the hydrophobic protein.

In conclusion, the complexes prepared here are depleted of LHC II but have a relatively intact electron transport which supports high rates of oxygen evolution without supplement of high concentrations of $Ca^{2+}$. Our complexes resemble the rice mutant preparations in many respects. However, our complexes have an important advantage over the rice mutant preparation that they can be isolated from normal plants. Thus the complexes are expected to serve as a useful material for investigation of the mechanism and structure of the oxygen-evolving machinery. In particular, we expect that our understanding on the function of $Ca^{2+}$ in oxygen evolution will be greatly advanced by the use of the complexes which show high rates of oxygen evolution in the presence of only one bound $Ca^{2+}$ but exhibit a strong $Ca^{2+}$ demand on removal of the extrinsic proteins. The complexes also would be a suitable material for topographic studies of the oxygen-evolving complexes by means of the crosslinking technique because they have all protein constituents needed for oxygen evolution but lack LHC II which strongly interferes with identification of proteins crosslinked [42,43].

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