

## Isolation and Characterization of an Oxygen-Evolving Photosystem II Reaction Centre Complex from Spinach\*

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The oxygen-evolving complex has often been considered as a discrete enzyme system separate from the PS II<sup>8</sup> reaction centre, but oxygen-evolving reaction centre preparations have recently been isolated from spinach thylakoids using digitonin<sup>1</sup> and from PS II membranes using octylglucoside.<sup>2</sup> In addition to the polypeptides in PS II core preparations,<sup>3</sup> these also contain the extrinsic 33 kDa protein.<sup>4</sup>

Here, the new reaction centre preparation with significantly higher oxygen-evolving activity is described. PS II membranes were solubilized with dodecylmaltoside in the presence of Ca<sup>2+</sup> and Cl<sup>-</sup> and fractionated by sucrose gradient centrifugation. The purified PS II complex contained 4.1 manganese atoms, 1.0 low-potential cytochrome *b*-559 and 0.9 Q<sub>A</sub> per 70 chlorophyll units, and was highly enriched in the intrinsic PS II reaction centre polypeptides and the extrinsic 33 kDa protein. It contained only trace amounts of the light-harvesting complex. The oxygen-evolving activity was more than 1200 μmol O<sub>2</sub> (mg Chl)<sup>-1</sup> h<sup>-1</sup>.

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<sup>8</sup>Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II; Q<sub>A</sub>, primary quinone acceptor of Photosystem II; Q<sub>B</sub>, secondary quinone acceptor of Photosystem II.

### Materials and methods

Oxygen-evolving (600 μmol O<sub>2</sub>(mg Chl)<sup>-1</sup> h<sup>-1</sup> with PPBQ as electron acceptor) PS II enriched membranes were prepared from spinach as described previously.<sup>5</sup> The PS II membranes were washed twice with 20 mM Mes-NaOH (pH 6.3), 25 mM NaCl and 300 mM sucrose and were suspended in 18 mM dodecylmaltoside, 20 mM Mes-NaOH (pH 6.3), 5 mM CaCl<sub>2</sub>, 15 mM NaCl and 300 mM sucrose at a level of 2.0 mg Chl ml<sup>-1</sup>. After incubation on ice for 20 min, the samples were centrifuged at 40 000 × *g* for 20 min to remove unsolubilized material and then loaded on 0.5–1.2 M sucrose gradients containing 20 mM Mes-NaOH (pH 6.3), 5 mM CaCl<sub>2</sub>, 15 mM NaCl and 1 mM dodecylmaltoside. The gradients were centrifuged in a Beckman type 70 Ti or 75 Ti fixed-angle rotor at 230 000 × *g* for 16 h at 5°C. The chlorophyll-containing bands were collected and concentrated by ultrafiltration with an Amicon XM100 Diaflo membrane.

The concentration of cytochrome *b*-559 was determined from chemically induced difference spectra<sup>6</sup> at room temperature. The concentration of the primary acceptor Q<sub>A</sub> was estimated from the light-induced reduction of Q<sub>A</sub><sup>7</sup> in samples containing 0.8 M Tris (pH 8.4) without addition of DCMU. The concentration of K<sub>3</sub>Fe(CN)<sub>6</sub> had to be reduced from 1 mM to 5 μM in a sample containing 0.13 mg Chl ml<sup>-1</sup> to avoid oxidation of Q<sub>A</sub> by ferricyanide in the light. Low temperature EPR measurements were performed with the equipment described in Ref. 8.

Activity measurements and manganese analyses were performed as described previously.<sup>5</sup> SDS-polyacrylamide gel electrophoresis was performed as described in Ref. 9 on 12–20% gradient gels in the absence of urea. The samples were incubated at room temperature for 1 h in 1% SDS, 4 M urea, 5% mercaptoethanol, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0).

## Results and discussion

In the sucrose gradients, 10–15% of the total chlorophyll was found in a pellet which contained incompletely solubilized material (Chl a/b ratio = 2.3). The upper band at 0.8–0.95 M sucrose had a Chl a/b ratio of 1.3 and contained primarily the light-harvesting complex. A band at 1.0–1.05 M sucrose, containing about 5% of the total chlorophyll, had a high Chl a/b ratio (8.1) and was highly enriched in polypeptides with molecular masses 55, 44, 34, 33 and 9 kDa (Fig. 1). The 55 and 44 kDa polypeptides correspond to the 47 and 43 kDa chlorophyll proteins, and the 34 kDa polypeptide corresponds to the intrinsic 34 kDa protein with probable role in manganese binding and oxygen evolution,<sup>10</sup> and/or to the herbicide-binding protein.<sup>11</sup> The 33 and 9 kDa polypeptides are identical to the extrinsic 33 kDa protein, which is known to stabilize manganese binding,<sup>4</sup> and the apoprotein of cytochrome *b*-559, respectively. The PS II complex contained only trace amounts of the 25–27 kDa polypeptides of the light-harvesting complex (Fig. 1). The polypeptide composition is similar to those reported in Refs. 1 and 2.

In the presence of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  the purified PS II complex evolved oxygen at a very high rate (Table 1), but was completely inactive in the absence of these ions. This requirement of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  for oxygen evolution could be traced to the

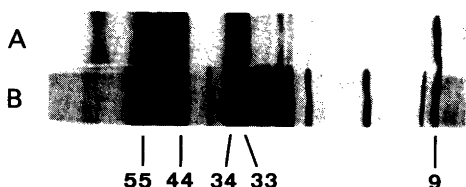


Fig. 1. Polypeptide analysis by SDS-polyacrylamide gel electrophoresis. (A) The oxygen-evolving PS II reaction centre complex. (B) Oxygen-evolving PS II membranes.

Table 1. Oxygen-evolving activity of the reaction centre complex and PS II membranes.<sup>a</sup>

Acceptor	RC complex		PS II membranes	
	control	+DCMU	control	+DCMU
PPBQ	752	80	614	0
DCBQ	1288	632	445	47
$\text{K}_3\text{Fe}(\text{CN})_6$	1272	608	144	0

<sup>a</sup>The assay medium contained 20 mM Mes-NaOH (pH 6.3), 10 mM  $\text{CaCl}_2$ , 400 mM sucrose and 1 mM electron acceptor. When indicated, DCMU (10  $\mu\text{M}$ ) was added. The activity is given in  $\mu\text{mol O}_2$  (mg Chl)<sup>-1</sup> h<sup>-1</sup>.

Table 2. Components of the oxygen-evolving reaction centre.

Component	Concentration
Manganese	4 per 68 Chl
Low potential cytochrome <i>b</i> -559	1 per 69 Chl
Primary acceptor $\text{Q}_A$	1 per 77 Chl

absence of the extrinsic 16 and 24 kDa subunits which normally serve to retain these ions.<sup>4</sup>

The affinity for electron acceptors was altered (Table 1), ferricyanide and DCBQ being the most efficient electron acceptors. DCMU (10  $\mu\text{M}$ ) inhibited 50% of this activity, while with PPBQ as electron acceptor 90% of the activity was inhibited by DCMU. This is in contrast to the reaction centre preparation of Ikeuchi *et al.*,<sup>2</sup> with which oxygen evolution was completely insensitive to DCMU. Furthermore, their preparation showed no activity with PPBQ as electron acceptor, indicating a complete block in the electron transfer between  $\text{Q}_A$  and  $\text{Q}_B$ .

The relative concentrations of the primary acceptor  $\text{Q}_A$ , cytochrome *b*-559 and manganese are shown in Table 2. The complex contained one cytochrome *b*-559 and four manganese atoms per reaction centre. The cytochrome was in the low-potential form (benzoquinone-oxidizable, ascorbate-reducible), probably due to the absence of the 16 and 24 kDa subunits (cf. NaCl-washed PS II membranes<sup>12</sup>).

With EPR spectroscopy, a multi-line spectrum corresponding to the  $\text{S}_2$  state of the oxygen-evolving system was observed (not shown). The hyper-

fine structure and the yield (on a reaction centre basis) were similar to those of the corresponding signal obtained for PS II membranes. This indicates that the donor side is quite intact in the reaction centre complex.

Summarizing, a PS II reaction centre complex has been obtained by solubilizing PS II membranes with dodecylmaltoside in the presence of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$ . The oxygen-evolving system seems to be quite intact, and the antenna size is less than 30% of the antenna size in PS II membranes. The preparation should be an excellent material for optical studies of the PS II donor side.

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