# An oxygen-evolving complex with a simple subunit structure – 'a water-plastoquinone oxidoreductase' – from the thermophilic cyanobacterium *Synechococcus* sp.

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An oxygen-evolving complex has been highly purified from the thermophilic cyanobacterium *Synechococcus* sp. The complex, which reproducibly showed 5 major polypeptide bands of 47, 40, 35, 30 and 9 kDa on SDS-polyacrylamide gel electrophoresis and contained 3.2 Mn per $Q_A$, had an oxygen-evolving activity of 300–400 μmol/mg chl per h in the presence of 5 mM $MnCl_2$ or $CaCl_2$. The complex most likely represents a minimum functional unit of the photosynthetic oxygen evolution.

*Oxygen evolution* *Photosystem II* *Reaction center complex* *Subunit structure* *Divalent metal cation* *Thermophilic cyanobacterium*

## 1. INTRODUCTION

A PS II preparation which is highly active in oxygen evolution was first isolated from the thylakoid membranes of the thermophilic cyanobacterium *Phormidium laminosum* after solubilization with lauryldimethylamine oxide [1]. Since then, oxygen-evolving preparations have been obtained from higher plants, algae and cyanobacteria using various detergents [2–9] and, together with inside-out thylakoid vesicles [10–12], greatly contributed to the recent advance in our understanding of the mechanism of photosynthetic oxygen evolution. In particular, the roles of 3 extrinsic proteins of 32–33, 23–24 and 16–17 kDa in water oxidation have been partially clarified [3,11–15]. However, these preparations provided relatively little information on the intrinsic proteins essential to oxygen evolution because they were fragments of the thylakoid membranes and hence contained large numbers of membrane proteins which are not directly related to the oxygen-evolving reaction. Recently, Yuasa et al. [16] isolated an interesting PS II reaction center complex from spinach which still retains 33 kDa protein and one Mn atom. The complex was, however, totally inactive in oxygen evolution.

Here, we report the isolation from the thermophilic cyanobacterium *Synechococcus* sp. of a PS II reaction center complex which can carry out electron transport from water to plastoquinone and hence represents a minimum functional unit of photosynthetic oxygen evolution.

*Abbreviations:* PS, photosystem; Mes, 2-(*N*-morpholino)ethanesulfonic acid; chl, chloroplyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

## 2. MATERIALS AND METHODS

An oxygen-evolving preparation was obtained from 2-day grown *Synechococcus* cells as in [17]. In short, thylakoid membranes (1 mg chl/ml) prepared with a medium containing 1 M sucrose, 50 mM Hepes-NaOH (pH 7.5), 10 mM NaCl and 5 mM $MgCl_2$ (1 M sucrose medium) were treated with 0.8% β-octylglucoside for 60 min at 25°C.



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After dilution with an equal volume of the same medium from which sucrose was omitted, 3–4 ml of the suspension was placed on 1.5 ml of 1 M sucrose medium and centrifuged at 300 000 × *g* for 60 min. A green band appeared at the interface between the 0.5 and 1 M sucrose layers and had a high activity of oxygen evolution [17]. This fraction, which we term the crude preparation, was applied to a Sepharose CL-4B column equilibrated with 1 M sucrose medium and eluted with the same medium. This step removed soluble proteins, especially phycobilins, from the green band which came off in the void volume. A significant amount of allophycocyanin was, however, still associated with the fraction eluted. To remove the bound phycobilin proteins, the green fraction (100 μg chl/ml) was incubated with 0.5% Na deoxycholate for 20 min at 25°C and placed onto a DEAE-cellulose column equilibrated with 1 M sucrose medium which contained 0.1% digitonin and, in place of Hepes buffer, 50 mM Mes-NaOH (pH 5.5). The charged column was washed with the same medium containing increasing concentrations of NaCl. A pink carotenoid band was eluted with 30–40 mM NaCl and solubilized allophycocyanin with 80 mM NaCl. A green band which was eluted with 0.75 M phosphate in the medium will be called the purified complex.

Polypeptide compositions were examined by SDS-polyacrylamide gel electrophoresis according to Laemmli [18]. Samples were incubated with 2.5% SDS, 5% 2-mercaptoethanol and 8 M urea for 30 min at 20°C, and then run on 12.5% slab gel containing 0.1% SDS and 6 M urea. The gels were stained with Coomassie brilliant blue and scanned with a Shimazu dual-wavelength chromatoscanner CS-910 at 560 nm.

Absorption spectra were measured with a Hitachi 320 spectrophotometer. Mn was determined with a Shimazu atomic absorption spectrophotometer AA-640-01 with a flameless graphite furnace. Oxygen evolution was monitored with a Clark-type oxygen electrode at 40°C under illumination with saturating white light. The basal reaction mixture contained 1 M sucrose, 50 mM Mes-NaOH (pH 5.5), 10 mM NaCl, 50 μM phenyl-*p*-benzoquinone and 1 mM ferricyanide. $Q_A$ was determined by measuring light-induced absorption changes at 325 nm with a Hitachi 356 spectrophotometer [17]. Samples were suspended in 1 M sucrose medium containing 200 μM ferricyanide and 10 μM DCMU. The differential extinction coefficient of $Q_A$ was assumed as 13 $mM^{-1} \cdot cm^{-1}$ [19].

## 3. RESULTS

The crude preparations have a high activity of oxygen evolution exceeding 1000 μmol $O_2$/mg chl per h [17]. The purified complexes were found to retain a capacity for evolving oxygen with phenyl-*p*-benzoquinone and ferricyanide as electron acceptors. The activity was lower than that of the crude preparations but still significant (table 1). The rate of oxygen evolution was about 100 μmol $O_2$/mg chl per h but increased usually to 300 and occasionally to 400 μmol $O_2$/mg chl per h on addition of 5 mM $MnCl_2$ or $CaCl_2$. The addition of 5 mM $MgCl_2$ was less effective. Thus, the enhancement of the activity can be ascribed to the divalent cations, rather than $Cl^-$, of the salts added. The stimulating effect of $Mn^{2+}$ and $Ca^{2+}$ was also observed with the crude preparations after EDTA treatment [17].

The activity of the purified complexes was strongly inhibited by DCMU. This indicates that phenyl-*p*-benzoquinone accepts electrons in the plastoquinone (or $Q_B$) region.

The purified complexes contained one Mn per 16 chl *a* (table 2). Because the complexes had one $Q_A$ (the secondary electron acceptor of PS II) per 51 chl *a*, there are 3.2 Mn per PS II reaction center. The crude oxygen-evolving preparations contain 4 Mn per PS II [17]. Thus the purified complexes still retained a major fraction of the functional Mn.

Table 1

Activities of the crude oxygen-evolving preparations and the purified complexes

| Preparations | Additions | Rates of $O_2$ evolution (μmol $O_2$/mg chl per h) |
|---|---|---|
| Crude preparations | – | 1200 |
| Purified complexes | – | 121 |
| Purified complexes | 5 mM $MnCl_2$ | 291 |
| Purified complexes | 5 mM $CaCl_2$ | 311 |
| Purified complexes | 5 mM $MgCl_2$ | 201 |

Table 2

The Mn and $Q_A$ contents of the purified complexes

| Components | mol chl *a*/mol |
|---|---|
| Mn | 15.9 ± 1.5 (3) |
| $Q_A$ | 50.5 ± 4.7 (3) |
| $Mn/Q_A$ | 3.2 |

Figures in parentheses indicate numbers of preparations examined

The absorption spectra of the crude preparation and purified complexes are illustrated in fig.1. It is seen that the purified complexes were almost completely free from phycocyanin and allophycocyanin which strongly absorb at 620 and 650 nm, respectively (fig.1). The absorption maxima of chl *a* occurred at 673 and 436 nm, and bands between 460 and 510 nm indicate the presence of carotenoids in the complexes.

Polypeptide compositions of the two oxygen-evolving preparations were examined by SDS-polyacrylamide gel electrophoresis (fig.2). The densitometry tracing of the crude preparation showed about 20 peaks or shoulders; major bands were 66, 60, 54, 51, 47, 44, 40, 35, 30, 27, 18, 16 and 14 kDa and a sharp band at the gel front. Prominent bands between 14 and 17 kDa indicate the association of a substantial amount of phycobilin protein subunits with the preparation. The purified complexes showed a much simpler polypeptide profile (fig.2B). Main polypeptides which appeared reproducibly in the purified complexes were 47, 40, 35 and 30 kDa polypeptides and the one at the gel front. A diffuse band of 18 kDa was seen in the sample used but its appearance was less reproducible.

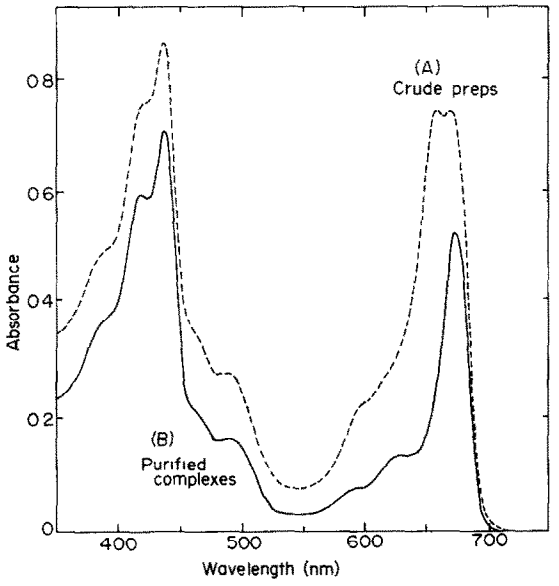


Fig.1. Absorption spectra of the crude preparations (A) and the purified complexes (B).

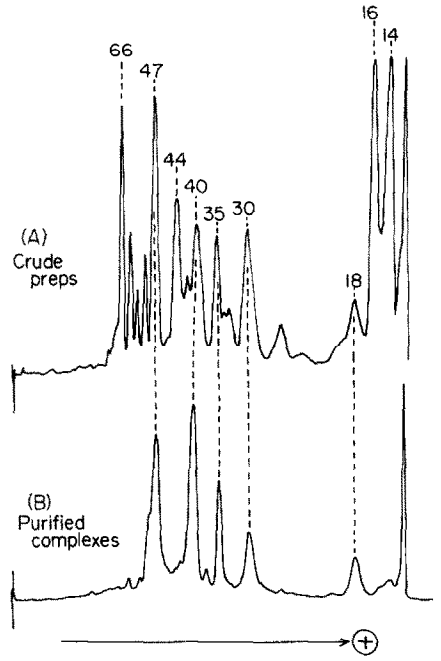


Fig.2. Densitometer tracings of polypeptides resolved by SDS-polyacrylamide gel electrophoresis from the crude preparations (A) and the purified complexes (B). Numbers indicate molecular masses in kDa.

Recently, a PS II reaction center complex was isolated and highly purified from *Synechococcus* and its constituent polypeptides were partially characterized [20–22]. The purified oxygen-evolving complexes have in common with the PS II reaction center complexes most of the polypeptides. The 47 and 40 kDa polypeptides are the apoproteins of a chlorophyll-protein which carries the site of the primary photochemistry and of another chlorophyll-protein which serves as an antenna of the reaction center complex, respectively [20,21]. The 47 kDa band was split into two bands in this particular preparation and hence ap-

pears shorter than the 40 kDa band as opposed to the situation in the PS II reaction center complexes. The 30 kDa band may be a composite of the 31 and 28 kDa bands of the reaction center complex. The tall band at the gel front cannot be explained by the apoprotein of cytochrome *b*-559 alone, indicating the presence of other small proteins of 9 kDa or less. The most important difference between the two PS II preparations was, however, the occurrence of the 35 kDa polypeptide in the purified oxygen-evolving complexes. The 35 kDa polypeptide most likely corresponds to the 33 kDa protein of higher plants which is essential for water oxidation [13,14], because the polypeptide was readily removed from the oxygen-evolving preparations by washing with a high concentration of Tris-HCl or $CaCl_2$ with concomitant loss of oxygen-evolving activity (not shown).

Two extrinsic proteins of 24 and 17 kDa are present in oxygen-evolving preparations from higher plants and play some roles in water oxidation [11–14, 23–26]. We have no evidence for the occurrence of the 24 kDa protein in the cyanobacterium (see also [27]). The purified complex apparently lacks this protein. It is doubtful that the 18 kDa polypeptide observed in the purified complexes (fig.2B) corresponds to the 17 kDa protein of higher plants because many other active preparations showed no 18 kDa polypeptide or only traces. The purified complexes contain a significant amount of a small protein(s) which co-migrates with the apoprotein of cytochrome *b*-559, but the contribution of such a small protein to water oxidation is not known. The oxygen-evolving system of the cyanobacterium appears to have a simpler subunit structure than does that of higher plants.

## 4. DISCUSSION

This work demonstrates that the capacity for photosynthetic oxygen evolution is located in a chlorophyll-protein complex with a simple subunit structure. The purified oxygen-evolving complex isolated here is in essence the PS II reaction center complex associated with the 35 kDa polypeptide. The complex contains 3 Mn atoms per $Q_A$ and shows oxygen evolution at rates of 300–400 $\mu$mol $O_2$/mg chl per h at 40°C. A preparation which has been purified from *Phormidium* [27] has 4 Mn per PS II and is more active in oxygen evolution than the *Synechococcus* complex. However, the *Phormidium* preparation still contains more than 10 polypeptides, indicating considerable protein contamination. The subunit structure of the PS II complex isolated from spinach is similar to ours but the complex contained only 0.6–1.0 Mn per PS II and is totally inactive in oxygen evolution [16]. Thus the *Synechococcus* complex is the most purified oxygen-evolving preparation so far reported.

The 33 kDa protein appears not to be a Mn-carrying protein [15]. Yuasa et al. [16], who isolated the spinach PS II complex, therefore concluded that one Mn atom binds to a subunit of the PS II reaction center complex. Our results further indicate that at least 3 Mn atoms are directly associated with constituent subunits of the PS II reaction center complex.

A putative biochemical entity which accumulates positive charges and eventually utilizes them to liberate oxygen and protons from water has often been referred to as the 'water-splitting enzyme' and its cooperation with the PS II reaction center complex was considered to be essential for electron transport from water to plastoquinone. The present work conclusively indicates that there is no such discrete protein complex functioning as the water-splitting enzyme and that the electron transport from water to plastoquinone is carried out by a single supramolecular complex. The function of the complex is analogous to that of NADH (succinate)-ubiquinone oxidoreductase of the mitochondrial respiratory chain which mediates electron transfer from NADH (succinate) to ubiquinone. Thus, the purified oxygen-evolving complex can be termed the water-plastoquinone oxidoreductase complex, or simply the water dehydrogenase complex. The complex has the unique capacity of utilizing photons as a substrate, which enables it to mediate the electron transport against a large redox potential difference.

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