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Chemical composition of purified oxygen-evolving complexes from the thermophilic cyanobacterium *Synechococcus* sp.

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Chemical constituents of a highly purified oxygen-evolving complex isolated from the thermophilic cyanobacterium *Synechococcus* sp. (Satoh, K., Ohno, T. and Katoh, S. (1985) FEBS Lett. 180, 326–330) was determined. Oxygen-evolving activity of the complex was considerably increased by improvements of purification procedures and the maximum rate of 1300 μmol O_2 per mg Chl per h was obtained at 40°C with ferricyanide as electron acceptor. Electrophoresis in polyacrylamide gradient gels resolved seven polypeptides of 47, 40, 35, 31, 28, 9 and 8 kDa from the complex. The light-harvesting pigment system of the complex consisted of 50 chlorophyll *a* and 7 β-carotene molecules but no xanthophylls. The complex contained two plastoquinone molecules, which may be attributed to the secondary electron carrier Q_A and possibly Z, because there were only low levels of Q_B remained unextracted. The complex contained 3.8 atoms Mn. A simple method was developed to determine bound Ca^{2+} in the complex without pretreatments to eliminate contaminating Ca^{2+} from chemicals and wares. The Ca content determined by this method varied from 0.46 to 0.93 atom with preparations. A good correlation found between the activity and the Ca content suggests that one bound Ca atom is essential for oxygen evolution. Five Fe atoms were present in the complex, of which two are explained by cytochrome *b*-559. In addition, a significant amount of fatty acid was associated with the complex. By assuming that each one of 47, 40, 35, 31, 28 and 8 kDa polypeptides and two 9 kDa polypeptide are present, the molecular weight of the complex was estimated to be $3 \cdot 10^5$.

Introduction

We have recently isolated a purified PS II complex competent in oxygen evolution with phenyl-*p*-benzoquinone and ferricyanide as electron acceptors from the thermophilic cyanobacterium *Synechococcus* sp. [1]. The complex which maximally evolves oxygen at a rate of 400 μmol O_2 per mg Chl per h contains Q_A, a bound plastoquinone functioning as a secondary electron acceptor of PS II, and 3.2 Mn atoms per 48 chlorophyll *a* molecules. SDS-polyacrylamide gel electrophoresis resolved five major polypeptides of 47, 40, 35, 30 and 9 kDa from the complex, of which the 47, 40, 30 and 10 kDa polypeptides are subunit constituents of the PS II reaction center complex [2,3]. The 35 kDa polypeptide which is solubilized by washing with high concentrations of Tris or $CaCl_2$ [1] corresponds to the extrinsic 33 kDa polypeptide of chloroplasts [4,5]. It was concluded that the water-plastoquinone oxidoreduc-

Abbreviations: PS, Photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DEAE, diethylaminoethyl; Mes, 4-morpholineethanesulfonic acid; Chl, chlorophyll.

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tase complex is in essence the PS II reaction center complex with an attached 35 kDa polypeptide and that there is no discrete protein complex specifically functioning in the water oxidation separately from the PS II reaction center complex.

Tang and Satoh [6] also have purifed a PS II oxygen-evolving complex from digitonin-solubilized spinach thylakoids by column chromatography. The spinach complex has simple subunit constituents similar to those of the cyanobacterial complex and evolves oxygen at a rate of about 150 μmol O_2 per mg Chl per h. More recently, Ikeuchi et al. [7] have isolated a highly active oxygen-evolving comple with a simple subunit structure from spinach PS II membranes using β-octylglucoside. The complex contains 4 Mn atoms per reaction center and shows the maximum oxygen-evolving rate of 850 μmol O_2 per mg Chl per h. Thus experiments from the three laboratories demonstrate that light-driven electron transport from water to plastoquinone takes place in a single multiprotein complex of the thylakoid membranes.

In the present work, we have analyzed various components of the *Synechococcus* oxygen-evolving complex in an attempt to determine the chemical composition of a minimum unit for oxygen evolution. Improvements of purification procedures considerably increased the oxygen-evolving activity of the purified complexes, yielding the maximum rate of 1300 μmol O_2 per mg Chl per h. Polypeptide composition and contents of Mn, Ca, Fe, plastoquinone and carotenoids have been determined. In particular, we have developed a simple assay method to determine Ca^{2+} in the complex which abbreviates laborious pretreatments to make chemicals and samples free from contaminating Ca^{2+}.

Materials and Methods

Synechococcus was autotrophically grown at 55°C for 2 days [8] and crude oxygen-evolving particles were prepared with β-octylglucoside according to the method of Satoh and Katoh [9]. The oxygen-evolving complexes were purified from the β-octylglucoside preparations as described in Ref. 1, except that the final stage of purification was carried out with DEAE-Toyopearl 650M (Toyo Soda) in place of DEAE-cellulose. In short, the β-octylglucoside particles were passed through a Sepharose CL-4B column, then incubated with 0.5% Na deoxycholate for 20 min. The suspension was placed onto a DEAE-Toyopearl column equilibrated with 1 M sucrose/50 mM MeS-NaOH (pH 5.5)/10 mM NaCl/5 mM $MgCl_2$/0.1% digitonin. After washing the charged column, the complexes were eluted with the same medium containing 0.75 M phosphate. The use of DEAE-Toyopearl greatly reduced amounts of chlorophyll remained uneluated at the top of the column. The column chromatography was carried out at 4°C as quickly as possible.

Oxygen evolution was determined with a Clark-type oxygen electrode at 40°C with saturating white light. The reaction medium contained 1 M sucrose/50 mM Mes-NaOH (pH 5.5)/10 mM NaCl/5 mM $MgCl_2$/1 mM ferricyanide. Q_A was determined spectrophotometrically by measuring absorption changes at 325 nm as described previously [10,11]. The differential extinction coefficient of Q_A used was 13 $mM^{-1} \cdot cm^{-1}$ [12]. Flash-induced absorption changes of Q_A at 415 nm was measured with a Union Giken single-beam spectrophotometer [13]. Cytochrome *b*-559 was determined by measuring the ferricyanide-oxidized minus dithionite-reduced difference absorption spectrum using the differential extinction coefficient of 16 $mM^{-1} \cdot cm^{-1}$ at 559 nm [14]. Polypeptide composition was analyzed as in Ref. 1 except that 10–15% polyacrylamide gradient gels were used.

Mn, Ca and Fe were determined with a Shimadzu atomic absorption spectrophotometer AA-640-01 with a flameless graphite furnace. Mn was determined by applying the sample suspension directly to the spectrophotometer. However, the determination of Ca was not so simple because all chemicals used for preparations and assay were contaminated with considerable amounts of the metal cation. Significant amounts of Ca^{2+} were detected in glass and plastic wares unless they were freshly and carefully cleaned. We have developed a simple method to determine Ca^{2+} in the oxygen-evolving complexes which abbreviates laborious procedures to eliminate contaminating Ca^{2+} from chemicals and wares used. To 1.0 ml of

the sample suspension containing 1 M sucrose/50 mM Mes-NaOH (pH 5.5)/10 mM NaCl/5 mM $MgCl_2$/0.5–1.0 mg chlorophyll, 0.5 g of a cation-chelating resin, Chelex 100 (sodium form, 100–200 mesh, Bio-Rad Laboratories) was added and the suspension was gently stirred for 5–10 min. After the resins were removed by centrifugation, the supernatant was applied to the spectrophotometer after dilution with 5 vols. of distilled-deionized water which had been further incubated with Chelex 100. The dilution was necessary because high concentrations of sucrose interfere with the atomic absorption spectrophotometry. Details of the procedures will be described elsewhere. Similar procedures were used for the determination of Fe.

Plastoquinone and carotenoids were extracted with acetone and separated with column and thin layer chromatography, respectively, as described previously [15]. Lipids were methanolysed with 5% HCl in methanol and resulting fatty acid methyl-esters were determined with a Shimadzu gas chromatograph GC-8A. Chlorophyll was estimated by the method of Mackinney [16].

Results

Oxygen-evolving activity

Rates of oxygen evolution in purified complexes prepared in the previous work were at most 400 μmols O_2 per mg Chl per h [1]. Checking the activity at each step of the purification, we found that rates of oxygen evolution were still high after the deoxycholate treatment, but drastically dropped during the DEAE-cellulose column chromatography. The activity of purified complexes was considerably improved by replacing DEAE-cellulose with DEAE-Toyopearl and by shortening duration of the column chromatography. The activity was somewhat variable with preparations: rates of oxygen evolution were usually 600–800 μmol O_2 per mg Chl per h, but often exceeded 1000 in the same unit at 40°C which is nearly 20° below the optimum temperature for photosynthesis in the cyanobacterium [17]. The maximum rate of 1300 μmol O_2 per mg Chl per h obtained corresponds to about 70% of the rate of the β-octylglucoside particles from which the complexes had been purified (Table I).

Oxygen evolution was routinely determined in the presence of 5 mM $CaCl_2$. The effect of Ca^{2+} was less pronounced with highly active preparations (Table I) as compared with the previous relatively inactive preparations, in which the rate of oxygen evolution was more than doubled with 5 mM $CaCl_2$ [1]. Mn^{2+}, which considerably enhanced the oxygen-evolving activity of the previous preparations [1], had no stimulating effect on highly active preparations (Table I). The assay medium also contained 20 mM Cl^- which is saturating for the activity. In contrast to the β-octylglucoside particles which require digitonin for ferricyanide photoreduction [9], oxygen evolution with ferricyanide as electron acceptor was not affected by the addition of the detergent in highly active complexes which had been prepared with digitonin. The activity was saturated with 1 mM ferricyanide. Phenyl-*p*-benzoquinone stimulated oxygen evolution in relatively inactive preparations, but not in highly competent preparations. Electron transport from water to ferricyanide was partially inhibited by DCMU (Table I). The extent of the DCMU inhibition varied considerably with preparations.

TABLE I

OXYGEN-EVOLVING ACTIVITY OF CRUDE AND PURIFIED COMPLEXES

Preparations	Additions	Rates of O_2 evolution (μmol O_2 per mg Chl per h)
Crude particles	–	1800
Purified complexes	–	1100
Purified complexes	5 mM $CaCl_2$	1300
Purified complexes	5 mM $MnCl_2$	1000
Purified complexes	10 μM DCMU	300

Polypeptide composition

In the previous experiments in which 12.5% acrylamide gels were used, five polypeptide bands of 47, 40, 35, 30 and 9 kDa molecular mass regions were resolved from the purified oxygen-evolving complexes [1]. The polypeptide profile of the complexes purified by the improved procedure was essentially the same as those of the previous preparations in the same gel system (not shown). The use of 10–15% acrylamide gradient gels

revealed, however, more detailed polypeptide composition of the complexes (Fig. 1). The 30 kDa band was split into two bands of 31 and 28 kDa in the gradient gel. The occurrence of two closely migrating bands in the 30 kDa region has been observed in the PS II reaction center complex [3] and the 28 kDa polypeptide was recently identified as the herbicide-binding protein of the cyanobacterium [18]. A band which migrates at the gel front in the 12.5% gel was too tall to be explained by the apoprotein of cytochrome *b*-559 alone and hence comigration of another small polypeptide of 9 kDa or less has been suggested [1]. The band was clearly resolved into two bands of 9 and 8 kDa in the gradient gel. The association of a polypeptide of this molecular mass class which is different from the cytochrome *b*-559 apoprotein has been reported with a cyanobacterial oxygen-evolving preparation [19]. Thus, the purified oxygen-evolving complex consists of seven polypeptides of 47, 40, 35, 31, 28, 9 and 8 kDa. Small amounts of other polypeptides resolved were contaminations [1].

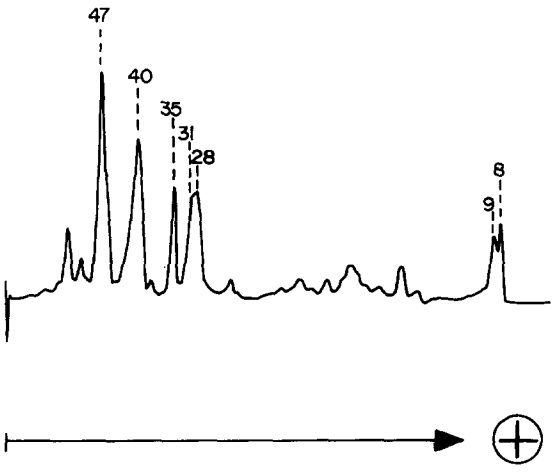

Fig. 1. Densitometer tracing of polypeptides resolved from purified oxygen-evolving complexes. The sample was incubated with 2.5% SDS/5% 2-mercaptoethanol/8 M urea for 30 min at 20°C, then run on a 10–15% acrylamide gradient gel containing 0.1% SDS/6 M urea. The gel was stained with Coomassie brilliant blue and scanned with a Shimadzu dual-wavelength chromatoscanner CS-910 at 560 nm. Numbers indicate molecular mass in kDa.

Q_A

Q_A was found at a ratio of 1 to about 50 chlorophyll *a* molecules in relatively inactive oxygen-evolving complexes prepared in the previous work [1]. Similar Q_A-to-chlorophyll ratios have been consistently obtained with highly active complexes, provided that β-octylglucosdie preparations containing negligible levels of P-700 had been used as starting materials. This indicates that not only Q_A, but also the primary photochemistry of PS II are resistant to the purification procedures used. In the following, the abundance of components determined was therefore expressed on the basis of Q_A.

Manganese

The previous estimation of the Mn content in the purified complexes showing oxygen evolution rates below 400 μmol O_2 per mg Chl per h yielded a mean value of 3.2 Mn atoms per Q_A [1]. The complexes more competent in oxygen evolution had higher Mn contents close to 4 atoms per Q_A (Table II). The results are consistent with the view that four Mn atoms per PS II reaction center are essential for oxygen evolution [20]. However, the ratios of near 4 were occasionally found in relatively inactive preparations, indicating that low rates of oxygen evolution cannot always be related to low Mn contents.

Calcium

Ca^{2+} is important cofactor of oxygen evolution [21] and in particular the requirement of Ca for PS

TABLE II

RELATIVE ABUNDANCE OF Mn, Ca, Fe, CYTOCHROME *b*-559 AND PLASTOQUINONE IN PURIFIED OXYGEN-EVOLVING COMPLEXES

Figures in parentheses indicate numbers of separate preparations examined.

Components	Mean (±SD) (mol/mol Q_A)
Mn	3.8 ±0.2 (6)
Ca	0.71 ±0.2 (5)
Fe	5.0 ±0.5 (3)
Cytochrome *b*-559	1.9 ±0.2 (5)
Plastoquinone	2.1 ±0.3 (6)

II electron transport in cyanobacteria has repeatedly been reported [22–25]. Determination of Ca^{2+} in biological materials is not so simple as that of Mn because various chemicals and glass wares are contaminated with significant amounts of the metal cation which more or less binds to organic compounds such as proteins and lipids. We found that the direct treatment of the purified complexes with Chelex 100 removes essentially all Ca^{2+} which is present in the suspending medium or is loosely bound to the complexes, thus enabling us to determine Ca^{2+} without time-consuming pretreatments to remove contaminating Ca^{2+} from chemicals, wares and samples. The abundance of Ca^{2+} thus determined varied from 0.46 to 0.93 atom per Q_A (Table II). There was a good correlation between the Ca^{2+} content and the activity: values above 0.9 were found in highly active preparations, whereas lower Ca contents were associated with low rates of oxygen evolution. The β-octylglucoside particles contained about one Ca^{2+} per Q_A. The results strongly suggest that one Ca atom which tightly bound to the complexes plays an important role in the PS II electron transport in the cyanobacterium.

Iron

The iron content of the purified complexes was determined after Chelex 100-treatment of the sample suspension. The Fe to Q_A ratios of about 5 were found in three preparations which showed considerably different rates of oxygen evolution (Table II). A similar Fe content has been reported with non-oxygen-evolving spinach PS II complexes [26]. A Triton particle, enriched in PS II (TSF-II) from spinach, contained two non-heme iron atoms per PS II reaction center [27].

Cytochrome b-559

Oxidation-minus-reduction difference spectra showed that two cytochrome *b*-559 are associated with the purified complex (Table II) in agreement with the previous experiments with more crude PS II preparations [28–30]. Thus two of the five Fe atoms associated with the complexes are present in the heme moiety of the cytochrome. About 30% of the cytochrome was hydroquinone reducible.

Plastoquinone and Q_B

About two plastoquinone molecules were found in the purified complexes per Q_A (Table II). Because the crude oxygen-evolving particles contain three plastoquinone molecules per Q_A [13], the purification procedures removed one plastoquinone molecule from the oxygen-evolving complexes. Q_A is totally oxidized by Q_B with an overall half time of several ms and the Q_A oxidation is inhibited by DCMU in the crude particles which have the secondary electron carriers, Q_A, Q_B and Z [13]. In contrast, Q_A is oxidized much more slowly and in a DCMU-insensitive manner in the PS II reaction center complexes which contain two plastoquinone molecules and have Q_A and Z but no Q_B [13]. Thus the ms and DCMU-sensitive oxidation of Q_A can be used to monitor the amount of functional Q_B remained unextracted in the purified complexes. The Q_B content of the purified complexes estimated varied from 0 to 50% of the total Q_A reduced by flashes. A fair correlation was found between the Q_B contents thus determined and numbers of plastoquinone molecules in excess of two molecules per Q_A.

Carotenoids

Synechococcus thylakoid membranes contain 11 β-carotene, 4 zeaxanthin, 4 myxoxanthophyll and 1 caroxanthin per 50 chlorophyll *a* [31]. Xanthophyll contents of the β-octylglucoside particles were lower and highly variable. Some preparations contained 2–3 molecules each of zeaxanthin and myxoxanthophyll per Q_A, whereas others totally lacked xanthophylls (Table III). No xanthophyll was detected in the purified complexes. β-Carotene seems to bind to the PS II complexes more strongly than do xanthophylls; about eight mole-

TABLE III

CAROTENOID CONTENT OF CRUDE AND PURIFIED OXYGEN-EVOLVING COMPLEXES

Preparations		β-Carotene	Zeaxanthin (mol/mol Q_A)	Myxoxanthophyll
Crude particles	(1)	8.0	2.4	3.1
	(2)	8.2	0	0
Purified complexes	(1)	7.4	0	0
	(2)	6.8	0	0

cules of β-carotene were associated with the crude preparations and only a small fraction of the carotenoid was solubilized during the purification of the oxygen-evolving complexes.

Discussion

Table IV summarizes the chemical composition of the purified oxygen-evolving complex. Numbers of molecule shown for respective components are not necessarily those actually determined but those deduced to be required for the oxygen-evolving activity. The PS II complexes highly competent in oxygen evolution are expected to contain all reaction components required for electron transport from water to plastoquinone, or to added electron acceptor, in sufficient quantities and a partial depletion of one of the essential components would result in low rates of oxygen evolution. Components which are not related, directly or indirectly, to the activity would be minimal, if any, in the highly purified complex. Thus the chemical composition shown in Table IV may represent that of a minimum functional unit for oxygen evolution.

TABLE IV

PROPOSED COMPOSITION OF A MINIMUM UNIT FOR OXYGEN EVOLUTION

Components	mol/mol Q_A	Molecular weight
Chlorophyll *a*	50	44700
β-Carotene	7	3760
Plastoquinone	2	1500
Mn	4	220
Ca	1	40
Fe	(5)	
Heme (Cyt *b*-559)	2	1240
Non-heme	3	170
		(51630)
Polypeptides		
47 kDa	1	47000
40 kDa	1	40000
35 kDa	1	35000
31 kDa	1	31000
28 kDa	1	28000
9 kDa	2	18000
8 kDa	1	8000
		(207000)
Fatty acids	120	44840
Sum		303470

The light-harvesting pigment system of the complex consists of 50 chlorophyll *a* and 7 β-carotene molecules. The β-carotene content should be regarded as the lower limit because the pigment might have been partially solubilized during the detergent treatment. In chloroplast, xanthophylls are associated with light-harvesting chlorophyll *a/b* proteins [32,33], whereas the location of xanthophylls in the cyanobacteria which lacks chlorophyll *b* is not known. The finding that the β-octylglucoside preparations occasionally contained xanthophylls suggest that a part of xanthophylls present in the thylakoid membranes is associates with the oxygen-evolving complexes and serves as a PS II antenna in situ.

The minimum number of plastoquinone molecule required for electron transport from water to ferricyanide is two, in agreement with the previous work with spinach PS II preparations [34]. One of the two bound plastoquinone molecules is ascribed to Q_A and another possibly to Z. Q_B is not essential for electron transport and ferricyanide is reduced directly by Q_A in the absence of Q_B. The activity of the purified complexes was partially inhibited by DCMU and the degree of the inhibition seems to be somewhat larger than the fraction of Q_B remained unextracted. However, more detailed experiments were needed to establish the relationship between the Q_B content and the DCMU-inhibition.

Of the three metal cations which are considered to be involved in the oxyge evolution, only the Mn^{2+} content in oxygen evolving PS II preparations has been established [20]. Much less is known about the abundance of Ca in PS II preparations mainly due to the difficulty in the accurate determination of the metal cation. It is therefore remarkable that the Ca^{2+} content of the PS II complex can be determined by a simple procedure to eliminate contaminating Ca^{2+} from sample suspensions with Chelex 100. The results obtained indicate that the competent oxygen-evolving complexes contain one Ca atom per Q_A. Recently, it has been shown that electron transport from Z to P-680 is specifically blocked by EDTA-treatment of the β-octylglucoside preparation in a hypotonic medium and the reactivation was accomplished by

incubating the inactivated samples with $CaCl_2$ [25]. It is highly likely that the bound Ca atom is involved in some way in this span of electron transport. Then there would be no bound Ca atom functioning in the water oxidation.

Of the five Fe atoms found in the purified complexes, two are explained by the heme moiety of cytochrome *b*-559 and one may be attributed to a Fe atom which is located close to Q_A [27,35]. Location and function of the rest should be studied in future. EPR studies suggest an electron transferring role of Fe on the oxidizing side of PS II reaction center [36].

The purified complexes contain seven subunits of 47, 40, 35, 31, 28, 9 and 8 kDa, of which five are attributed to the PS II reaction center complex [3]. The role of the 8 kDa polypeptide newly resolved remains to be studied (see Ref. 19). The relative abundance of the subunit polypeptides is not known. We tentatively assume stoichiometry of the subunit polypeptides as shown in Table IV.

Table IV also includes results of a preliminary experiment that the purified complex contains about 120 fatty acids per Q_A. Thus the complex is a lipoprotein complex, although the abundance of fatty acids in the complex corresponds to only 10% and 20% of the fatty acid contents of the thylakoid membranes and the β-octylglucoside particles, respectively. Assuming that complex contains only monogalactosyldiglyceride in which two thirds of the fatty acids are palmitic acids and the rest is oleic acids, the molecular weight of the complex was estimated as $3 \cdot 10^5$. The molecular sieve chromatography with a Sepharose CL-6B column gave a larger value of about $5 \cdot 10^5$ for an apparent molecular weight of the purified complex (not shown). The difference may be attributed to association of detergents with the complex. In addition, the total molecular weight of polypeptides would be an underestimation because molecular weight of membrane proteins determined by SDS-gel are often considerably smaller than those estimated from the amino acid sequence [37]. The results demonstrate that the minimum unit for oxygen evolution is a large discrete multiprotein complex with an extremely complex chemical composition.

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