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Inhibition by ethylenediamine tetraacetate and restoration by Mn^{2+} and Ca^{2+} of oxygen-evolving activity in Photosystem II preparation from the thermophilic cyanobacterium, *Synechococcus* sp.

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The Photosystem II particles capable of evolving oxygen with ferricyanide as an electron acceptor were isolated from β-octylglucoside-solubilized thylakoid membranes of the thermophilic cyanobacterium *Synechococcus* sp. High and steady rates of oxygen evolution were observed only in the presence of 0.5% digitonin and 1 M sucrose. The activity was totally lost when the particles had been treated with 1 mM ethylenediamine tetraacetate for 1 min in a hypotonic medium. The treatment removed two out of four Mn atoms present per the Photosystem II reaction center in the particles. A significant rate of oxygen evolution was restored almost immediately after the addition of 5 mM $MnCl_2$ to the treated particles. The activity was also slowly and partially restored when the treated particles had been incubated with 5 mM $CaCl_2$. $MgCl_2$ was much less effective irrespective of the incubation time. The addition of $MnCl_2$ to the $CaCl_2$-restored particles increased the activity to a level which is significantly larger than the sum of the $MnCl_2$- and the $CaCl_2$-induced increases in the activity. The Mn- and Ca-effects were largely suppressed by the simultaneous addition of $CaCl_2$ and $MnCl_2$, respectively. It is concluded that both Mn^{2+} and Ca^{2+} are essential components of the water oxidation of photosynthesis, but the two cations support oxygen evolution through different mechanisms.

Introduction

The involvement of Mn in the water oxidation of photosynthesis has been suggested from earlier observations that oxygen evolution is suppressed in Mn-deficient algal cells and plant leaves, but is restored on addition of Mn salts (for reviews, see Refs. 1–3). More recently, the reactivation of the Photosystem II electron transport by Mn has been demonstrated in cell-free systems. Yamashita et al. [4] showed that oxygen evolution is stimulated slightly on addition of $MnCl_2$ to chloroplasts, from which Mn had been partially removed by Tris treatment. Reconstitution of the primary charge separation of Photosystem II and photoreduction of DCIP by Mn^{2+} has been reported by Klimov et al. [5] with Tris-treated Photosystem II particles. DCIP photoreduction in the inside-out thylakoid vesicles was partially suppressed by EGTA but was restored by divalent cations including Mn^{2+} [6].

There are reports suggesting a possible function of Ca^{2+} in Photosystem II electron transport in cyanobacteria [7,8]. Ca^{2+} stimulated photoactivation of oxygen evolution in dark grown spruce

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; EDTA, ethylenediamine tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Chl, chlorophyll.

chloroplasts [9]. Requirement for not only Mn^{2+} but also Ca^{2+} of light-dependent activation of the water oxidation has been reported in Tris-acetone-treated chloroplasts [10,11], in intact chloroplasts from wheat which had been grown under intermittent light [12], and in *Anacystis nidulans* cells which had been lyophilized, treated with lysozyme, then extracted with EDTA [13]. Recently, the reconstruction of oxygen evolution by Ca^{2+} were demonstrated with Photosystem II particles which had been inactivated by treatments with high concentrations of NaCl [14] or $CaCl_2$ [15].

In the present work, we report preparation of oxygen-evolving Photosystem II particles from β-octylglucoside-solubilized thylakoid membranes of the thermophilic cyanobacterium, *Synechococcus* sp. Oxygen evolution was strongly inhibited by incubating the particles with EDTA in a hypotonic medium but the addition of Mn^{2+} and Ca^{2+} to the EDTA-treated particles restored a substantial activity of oxygen evolution. The results indicate that both Mn and Ca are essential components of the water oxidation system of photosynthesis.

Materials and Methods

The thermophilic cyanobacterium, *Synechococcus* sp., was grown for 2 days at 55°C as described previously [16,17]. Washed cells were incubated with 0.1% lysozyme and passed through a French Presser cell [18]. After treatment with DNAase, the thylakoid membranes were collected by centrifugation and washed once to remove phycobiliproteins. The isolation medium comprised 50 mM Hepes (pH 7.5)/10 mM NaCl/1 M sucrose. The thylakoid membranes were finally suspended in the medium comprising 1 M sucrose/10 mM NaCl/5 mM $MgCl_2$/50 mM Hepes (pH 7.5) (1 M sucrose medium).

Oxygen evolution was measured at 45°C using a Clark-type oxygen electrode [19]. The basal reaction mixture contained 1 M sucrose/10 mM NaCl/5 mM $MgCl_2$/50 mM Mes (pH 5.5)/1 mM ferricyanide/0.5% digitonin. An Ushio halogen lamp (650 W) was used as a source of saturating actinic light ($6.5 \cdot 10^2$ J · m^{-2} · s^{-1}). P-700 and Q (X320) were estimated from light-induced absorbance changes at 700 and 325 nm, respectively, with a Hitachi 356 Spectrophotometer [20,21]. The extinction coefficients used for P-700 and X320 were 64 [22] and 13 mM^{-1} · cm^{-1} [23], respectively. The Mn content was measured with a Shimazu Atomic Absorption Spectrophotometer type AA-640-01 equipped with a graphite furnace atomizer, GFA-2. Chlorophyll *a* was determined according to the method of Mackinney [24].

Results

Isolation of oxygen-evolving Photosystem II particles

The thylakoid membranes (1.0 mg chlorophyll/ml) isolated from *Synechococcus* sp. were incubated with 0.8% β-octylglucoside in the 1 M sucrose medium at room temperature (25°C) for 1 h. After the sucrose concentration was decreased to 0.5 M by adding an equal volume of the medium containing no sucrose, 3–4 ml of the suspension was placed on 1.5 ml of the 1 M sucrose medium and centrifuged at 300 000 × *g* for 60 min. Five fractions separated after centrifugation were called fraction 1, 2, 3, 4 and 5 from top downward. Fraction 1 was only faintly colored, whereas fraction 2 was blue, showing the presence of large amount of phycobiliproteins. Fraction 3, a band appeared at the boundary of 0.5 and 1 M sucrose and fraction 4 in the 1 M sucrose medium were blue-green or green in color. The precipitates (fraction 5) mainly consisted of membrane fragments.

Table I shows distribution of chlorophyll *a* and photochemical activities among the five fractions separated. Fraction 1 contained a small amount of chlorophyll *a*, whereas 80–90% of chlorophyll *a* was recovered in fraction 5. The yield of fraction 3 plus 4 was about 10% on the chlorophyll base. P-700 was practically undetectable in fraction 3 and was present in a very small amount (0–1.5 P-700 per 1000 chlorophyll) in fraction 4. The two fractions showed high rates of photosynthetic oxygen evolution with ferricyanide as electron acceptor. By contrast, fraction 5 was enriched in P-700 and totally inactive in the Photosystem II activity.

Thus, the β-octylglucoside treatment, combined with the cushion centrifugation, is a simple but excellent method to separate Photosystem I and II from *Synechococcus* thylakoid membranes without

TABLE I

DISTRIBUTION OF CHLOROPHYLL *a*, P-700 AND OXYGEN-EVOLVING ACTIVITY AMONG FIVE FRACTIONS OBTAINED FROM β-OCTYLGLUCOSIDE-SOLUBILIZED THYLAKOID MEMBRANES

Oxygen evolution was determined at 45°C with a Clark type oxygen electrode in the medium comprising 1 M sucrose/10 mM NaCl/5 mM $MgCl_2$/50 mM Mes (pH 5.5)/1 mM ferricyanide/0.5% digitonin.

Fraction	Chlorophyll (%)	P-700 / 1000 Chl	Rate of O_2 evolution (μmol O_2 per mg Chl/h)
1	0.3	0	0
2	4.1	0	418
3	9.5	0	886
4	2.2	0.4	806
5	83.5	7.9	0

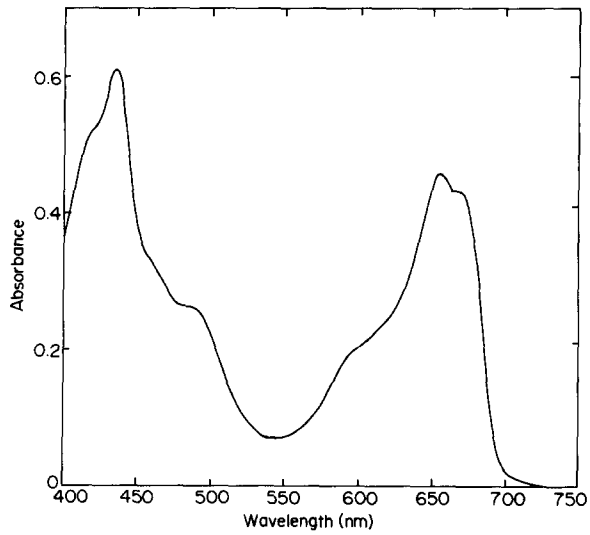

Fig. 1. An absorption spectrum of fraction 3.

destroying the oxygen-evolving capacity. We used fraction 3 throughout the following experiments because this fraction has the highest activity of oxygen evolution but negligible contamination of Photosystem I. Occasionally, more than 10% of chlorophyll *a* was recovered in fraction 3 with a significant contamination of P-700. Good separations such as those shown in Table I were obtained, however, by decreasing slightly the detergent concentration. The oxygen-evolving Photosystem II particles suspended in the 1 M sucrose medium still showed a substantial activity after the storage at −20°C for a month. The absorption spectrum of fraction 3 is illustrated in Fig. 1. A peak at 655 nm indicates that a substantial amount of allophycocyanin is still associated with the fraction.

Characteristics of the oxygen-evolving activity

The Photosystem II particles (fraction 3) showed high rates of oxygen evolution ranging from 300 to 1300 μmol oxygen evolved per mg chlorophyll per h with ferricyanide as an electron acceptor. However, ferricyanide can support a high and steady rate of oxygen evolution only in the presence of digitonin. As shown in Fig. 2, the activity was very low in the absence of the detergent and the maximum rate of oxygen evolution was attained in the presence of 0.5–0.6% digitonin. Duroquinone, 2,6-dichloro-*p*-benzoquinone and DCIP were poorer Hill oxidants whether or not digitonin was present. The optimum pH was between 5.0 and 5.5. The oxygen-evolving activity was, therefore, determined in the presence of 1 mM ferricyanide and 0.5% digitonin at pH 5.5 throughout the present work.

The presence of a high concentration of sucrose in the assay medium was necessary for the Photosystem II particles to exhibit high rates of oxygen evolution. The activity was low in the absence of

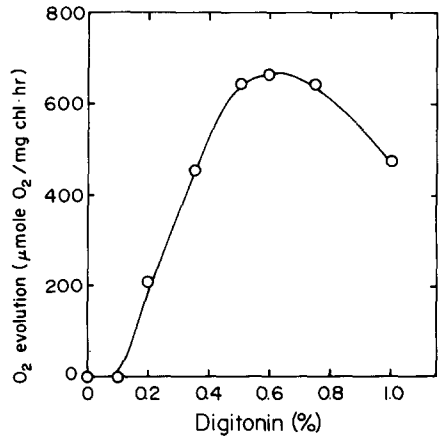

Fig. 2. Dependence on the digitonin concentration of oxygen-evolving activity in fraction 3. Oxygen evolution was determined as described in Table I except that the concentration of digitonin was varied as indicated. Chlorophyll *a* concentration was 3.1 μg/ml.

sucrose but increased with increasing concentrations of the sugar in the assay medium to reach the maximum level at about 1 M (Fig. 3). This suggests that the oxygen-evolving activity strongly depends upon the conformation of the Photosystem II particles which is sensitive to the osmolarity of the assay medium.

Fig. 3 shows that the activity determined in the assay medium containing 0.1 M sucrose was less than a half of the maximum activity at 1 M sucrose. The prior incubation of the particles in 0.1 M sucrose for 5 min, however, did not give rise to any appreciable inactivation provided that the activity was determined in the presence of 1 M sucrose. Thus, the lowering of the activity in a hypotonic reaction medium cannot be related to an irreversible release from the particles of a component(s) which is essential for the oxygen evolution.

Effects of EDTA

The activity of the oxygen-evolving particles was little affected by EDTA in the medium containing 1 M sucrose, whereas the incubation of the particles with EDTA in a hypotonic medium caused a marked inactivation of oxygen evolution. In the experiments shown in Fig. 4, the particles

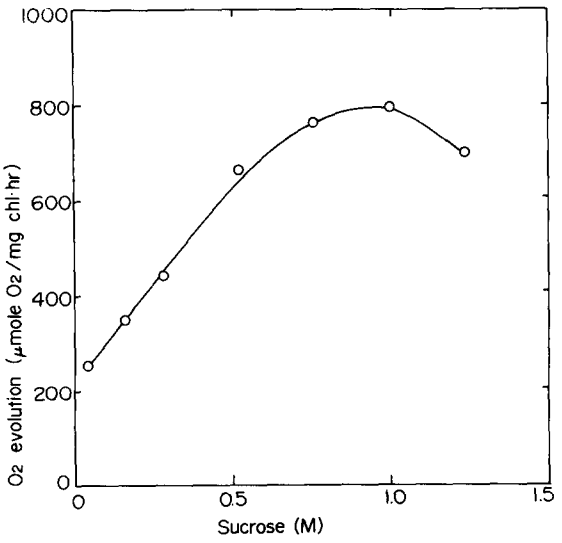

Fig. 3. Dependence of oxygen evolution on the sucrose concentration. Oxygen evolution was determined as in Table I, except the sucrose concentration was varied as indicated. Chlorophyll *a* concentration was 2.8 μg/ml.

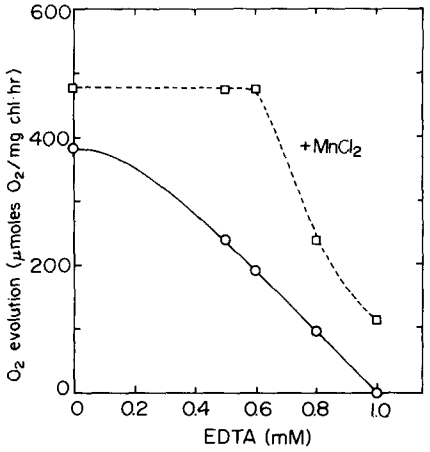

Fig. 4. Inhibition by EDTA and restoration by $MnCl_2$ of oxygen-evolving activity. The Photosystem II particles were incubated with indicated concentrations of EDTA for 1 min at 25°C in the medium contained 0.1 M sucrose and 5 mM Hepes (pH 7.5), then 5 mM $MgCl_2$ was added to remove free EDTA. The oxygen evolution was determined as described in Table I. Chlorophyll *a* concentration was 5.1 μg/ml. Where indicated, 5 mM $MnCl_2$ was added. The activity before the EDTA-treatment was 378 μmoles O_2 per mg Chl/h.

were incubated with various concentrations of EDTA in the medium containing 0.1 M sucrose for 1 min at 25°C, and after the addition of an excess of $MgCl_2$ to prevent further development of the EDTA effect, the activity was measured in the presence of 1 M sucrose. The activity was half reduced at 0.6 mM and completely inhibited at 1.0 mM EDTA. As stated above, the short exposure of the particles to 0.1 M sucrose itself had no effect on the activity. It appears, therefore, that a conformation change caused in the hypotonic medium is necessary to make the particles sensitive to EDTA.

In order to examine the cause of the inactivation by the chelator, we determined the Mn contents of the particles before and after the EDTA-treatment (Table II). The untreated particles contained 1 Mn atom per 13.5 chlorophyll *a* molecules. We also estimated the content of Q, the secondary electron acceptor of Photosystem II, in the particles by measuring absorbance changes at 325 nm. There was one Q per every 48 chlorophyll molecules, yielding a value of about four Mn atoms per Photosystem II reaction center. This agrees with the Mn contents of other Photosystem II particles [25–28].

TABLE II

CONTENTS OF Q AND Mn IN THE OXYGEN-EVOLVING PARTICLES

Components	Chlorophyll *a*/component	Mn/Q
Mn (untreated)	13.5 ± 1.7 (6)	3.5
Mn (EDTA-treated)	24.2 ± 1.9 (4)	2.0
Q	47.5 ± 6.8 (6)	

After the incubation of the particles with 1 mM EDTA in the hypotonic medium, the Mn content decreased to 24.2 ± 1.9 chlorophyll *a* per Mn, or about two Mn atoms per Photosystem II reaction center. We concluded, therefore, that EDTA inactivates oxygen evolution by removing a fraction of Mn which is essential for the activity from the particles.

Effects of Mn^{2+} and Ca^{2+}

Our conclusion was supported by the finding that the addition of $MnCl_2$ to EDTA-treated particles caused a significant restoration of the oxygen-evolving activity. Fig. 4 shows that the activity of untreated particles was somewhat stimulated by the addition of 5 mM $MnCl_2$ but the effect of $MnCl_2$ was more pronounced in partially inactivated preparations. It is seen that the activity of the particles treated with 0.6 mM EDTA was fully restored in the presence of $MnCl_2$. Although the extent of the restoration decreased at higher EDTA concentrations, a significant reactivation was still observed in the particles which had been completely inactivated with 1 mM EDTA. Fig. 5 shows that the reactivation of 1 mM EDTA-treated samples was maximal at 5 mM $MnCl_2$, and higher concentrations of $MnCl_2$ were rather inhibitory. We noticed that, the higher the original activity of the particles is, the larger the extent of the reactivation. The rates of oxygen evolution restored by 5 mM $MnCl_2$ were 100 μmol O_2 per mg Chl/h or less with the particles which had the activity ranging from 300 to 500 μmol O_2 per mg Chl/h before the EDTA-treatment, whereas oxygen evolution up to 300 μmol O_2 per Chl/h was restored with the preparations having the original activity of about 1000 μmol O_2 per mg Chl/h.

$CaCl_2$ also was found to increase the oxygen-evolving activity of EDTA-treated particles. There was a notable difference in the mode of the reactivation between $MnCl_2$ and $CaCl_2$. The effect of $MnCl_2$ fully developed within 1 min of the incubation, which was needed to equilibrate the reaction mixture thermally. In contrast, the preincubation of the EDTA-treated particles with $CaCl_2$ for 4–5 min was necessary to attain the maximum extent of the reactivation (Fig. 6). The reactivation was maximal at 5 mM $CaCl_2$ (Fig. 7). The extent of the reactivation again varied depending on the original activity of the particles used.

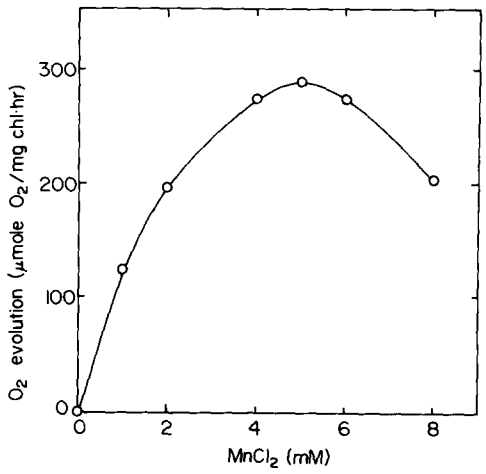

Fig. 5. Restoration of the oxygen evolution activity of EDTA-treated particles as a function of $MnCl_2$ concentration. The Photosystem II particles were incubated with 1 mM EDTA for 1 min as described in Fig. 4, but 5 mM $MgCl_2$ was not added to remove free EDTA. Instead, aliquots were added to the reaction media containing $MnCl_2$ at indicated final concentrations and oxygen evolution was measured immediately after the thermal equilibrium was attained. Chlorophyll *a* concentration was 2.3 μg/ml. The activity before the EDTA-treatment was 780 μmoles O_2 per mg Chl/h.

The results presented in Table III indicate that $MnCl_2$ and $CaCl_2$ reactivate the oxygen evolution by different mechanisms. The first column of Table III compares the effects of $CaCl_2$, $MnCl_2$ and $MgCl_2$ on the activity of EDTA-treated particles which was determined immediately after the thermal equilibrium had been attained. The largest restoration was attained with $MnCl_2$ followed by $CaCl_2$, whereas $MgCl_2$ was only slightly effective. This indicates that the restoration is caused by cations, but not by Cl^- of the salts added. The

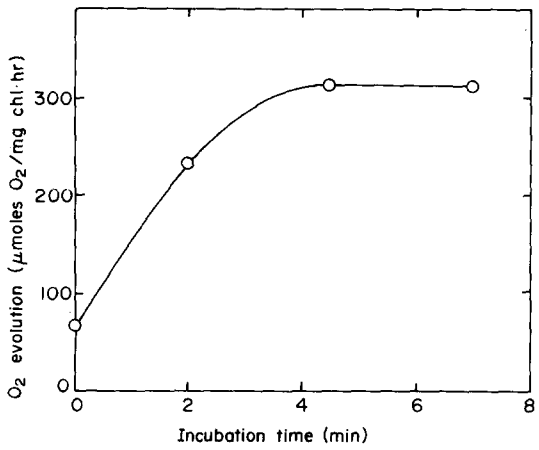

Fig. 6. Reactivation of oxygen evolution as a function of the incubation time of the EDTA-treated particles with $CaCl_2$. Experimental procedures were as in Fig. 5, except that the reaction media contained 5 mM $CaCl_2$ instead of $MnCl_2$ and oxygen evolution was measured after indicated periods of the incubation time. Chlorophyll *a* concentration was 2.3 μg/ml. The activity before the EDTA-treatment was 780 μmol O_2 per mg Chl/h.

Ca^{2+} effect observed may be ascribed to a partial progress of the slow-developing reactivation by the cation (see Fig. 6). We conclude, therefore, that the fast reactivation of oxygen evolution is highly specific to Mn^{2+}.

The effects of three cations on the activity of

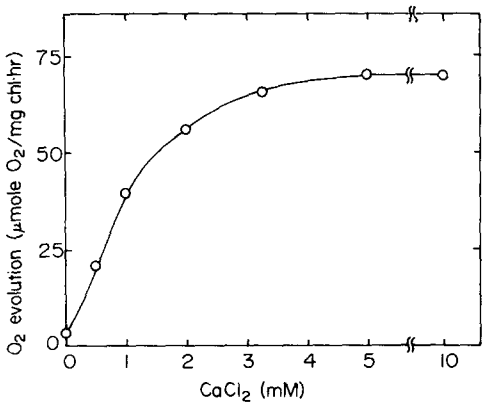

Fig. 7. Reactivation of oxygen evolution as a function of $CaCl_2$ concentration. Experimental procedures were as in Fig. 6, except that $CaCl_2$ concentration was varied and the incubation time was 5 min. Chlorophyll *a* concentration was 3.8 μg/ml. The activity before the EDTA-treatment was 342 μmoles O_2 per mg Chl/h.

TABLE III

EFFECTS OF DIVALENT CATIONS ON THE RECOVERY OF THE OXYGEN-EVOLVING ACTIVITY

Experimental procedures were as in Figs. 5 and 6. The activity was assayed immediately after the thermal equilibrium was attained (first column), or after incubation with 5 mM $CaCl_2$ (second column), 5 mM $MnCl_2$ (third column) or 5 mM $MgCl_2$ (fourth column) for 5 min. Where indicated, 5 mM of $CaCl_2$, $MnCl_2$ or $MgCl_2$ was added to the assay medium. The assay media in the 2nd–4th columns also contain 1 mM of the cations used for the preincubation. The concentration of chlorophyll *a* in the assay medium was 2.6 μg/ml. The activity before the EDTA-treatment was 333 μmoles O_2 per mg Chl/h.

Additions	Rate of oxygen evolution (μmol O_2 per mg Chl/h) EDTA-treated particles incubated with			
	None	5 mM $CaCl_2$	5 mM $MnCl_2$	5 mM $MgCl_2$
None	0	122	64	51
5 mM $CaCl_2$	26	141	109	51
5 mM $MnCl_2$	71	224	90	96
5 mM $MgCl_2$	10	135	51	39

the EDTA-treated particles, which have been incubated with 5 mM $CaCl_2$ for 5 min, are shown in the second column. The rate of oxygen evolution was 122 μmol O_2 per mg Chl/h after the preincubation with $CaCl_2$, but increased further to 224 in the same unit on addition of $MnCl_2$, which is more than the sum of the rate of oxygen evolution attained by $MnCl_2$ alone (the first column) and that attained by $CaCl_2$ alone. The synergistic effect of the two cations clearly indicates that Mn and Ca restore the activity through different mechanisms. Addition of $CaCl_2$ or $MgCl_2$ to the Ca-incubated particles were much less effective.

The top row of Table III shows that the restoration of the activity is specific to Ca^{2+} when determined after the 5 min preincubation. The effect of Mn^{2+} observed here should at least partly be ascribed to $MnCl_2$ which has been carried over to the assay medium to a final concentration of 1 mM. The observation that $MgCl_2$ was similarly effective suggests, however, that there is an unspecific divalent cation-effect to restore the activity to a limited extent.

The order of the addition of Mn^{2+} and Ca^{2+} is crucial to observe the synergistic effect of the two cations (Table IV). It is seen that the extent of the

TABLE IV

EFFECTS OF SIMULTANEOUS ADDITIONS OF $MnCl_2$ AND $CaCl_2$ ON THE Mn^{2+}- AND THE Ca^{2+}-DEPENDENT REACTIVATION OF OXYGEN EVOLUTION

Experimental procedures were as in Figs. 5 and 6. (A) Oxygen evolution of the EDTA-treated particles was measured in the presence of $MnCl_2$ alone, or with $MnCl_2$ and $CaCl_2$, each at 5 mM. (B) EDTA-treated particles were incubated with $CaCl_2$ alone, or with $CaCl_2$ and $MnCl_2$, each at 5 mM, for 5 min, then the activity was determined in the presence of 5 mM $MnCl_2$. The activity before the EDTA-treatment was 358 μmol O_2 per mg Chl/h.

Additions and treatments	Rate of O_2 evolution (μmoles O_2 per mg Chl/h)
A + $MnCl_2$	72
+ $CaCl_2$ plus $MnCl_2$	5
B incubation with $CaCl_2$	266
incubation with $CaCl_2$ plus $MnCl_2$	153

reactivation occurred on addition of $CaCl_2$ to the $MnCl_2$-incubated particles was considerably less than the extent of the reactivation observed on addition of $MnCl_2$ to the $CaCl_2$-incubated particles. This again stresses the importance of the 5 min preincubations for the $CaCl_2$-induced restoration.

Table IVB further shows that the preincubation of the EDTA-treated particles with both $CaCl_2$ and $MnCl_2$ was much less effective in restoring the activity than the preincubation with $CaCl_2$ alone. Evidently, the presence of Mn^{2+} in the preincubation medium suppresses the development of the Ca^{2+} effect. Similarly, $CaCl_2$ inhibits the Mn-dependent fast reactivation of the oxygen evolution. No significant reactivation occurred on the combined addition of 5 mM $MnCl_2$ and 5 mM $CaCl_2$ to EDTA-treated particles, whereas the addition of 5 mM $MnCl_2$ alone gave rise to a partial reactivation of the oxygen evolution. These results indicate that the binding of Mn^{2+} and Ca^{2+} to their respective specific sites is a prerequisite for the reactivation of oxygen evolution and that the two divalent cations compete for each binding site.

Discussion

In the present work, a Photosystem II preparation with a high activity of oxygen evolution was isolated from the thermophilic cyanobacterium, *Synechococcus* sp., with the use of a nonionic detergent, β-octylglucoside. The oxygen-evolving Photosystem II particles have been isolated from the same cyanobacterium by Miyairi and Schatz [29], with LDAO and by Schatz and Witt with sulfobetaine [30]. The three preparations share common features such as the association of phycobiliproteins with the particles, and the high-temperature dependence and an acidic optimum pH of oxygen evolution. Ferricyanide photoreduction in the sulfobetaine particles is also enhanced in the presence of the detergent [31].

There is, however, a notable difference in the antenna size of Photosystem II among the particles. The β-octylglucoside particles contain 48 chlorophyll *a* molecules per Q. Photosystem II particles from other cyanobacteria also contain one Photosystem II reaction center per 20–50 chlorophyll *a* [6,25,27,28]. In contrast, the chlorophyll *a*/Photosystem II reaction center ratio is 100 in the sulfobetaine particles and even larger in the LDAO particles [29,30]. We suspect that there might be an important difference in the culture conditions employed by the two groups because the ratio of chlorophyll *a* to Photosystem II reaction center is 400 in the cells grown in our laboratory [32] and 1000 in the thylakoid membranes employed by Schatz's group [30]. The antenna size of Photosystem I in our thylakoid membranes is again about 2-fold smaller than those in theirs.

The octylglucoside particles are highly sensitive to EDTA and totally inactivated after the incubation with 1 mM EDTA for 1 min in the hypotonic medium. About four Mn atoms are associated with the Photosystem II reaction center in untreated particles and the EDTA-treatment resulted in the extraction of about half of the bound Mn atoms from the particles. Thus the inactivation of oxygen evolution can be ascribed to the removal of a part of manganese which is essential for oxygen evolution. The results are in line with previous observations that four Mn atoms are associated with one Photosystem II reaction center in the particles active in oxygen evolution [25–28] and that oxygen evolution is linearly related to a part of manganese atoms which are more readily extracted by various treatments of algal cells, chloroplasts or Photosystem II preparations [25,33,34].

The most remarkable finding obtained in the present work is that a substantial activity of oxygen evolution is restored on addition of Mn^{2+} to EDTA-inactivated particles. The reactivation is specific to Mn^{2+} because Mg^{2+} and Ca^{2+} are much less effective in the rapid restoration of the activity. The results strongly support the view that Mn is an essential component in the oxygen-evolving system.

Mn-dependent activation of oxygen evolution, or electron transport from water to added acceptors, has been reported in Mn-deficient algal cells [35], Tris-acetone-treated chloroplasts [10,11] or intact chloroplasts isolated from wheat grown under intermittent illumination [12]. In these cases, the activation proceeds only slowly and exclusively under illumination, reflecting a complexity of the processes involved. In contrast, the oxygen-evolving activity developed almost immediately after the addition of Mn^{2+} to EDTA-treated particles in the dark. This suggests that the rebinding of Mn^{2+} to its specific functional sites is the cause of the reactivation of the EDTA-treated particles.

Klimov et al. [5] reported that Tris-washing extracts practically all bound Mn from Triton Photosystem II particles and that the addition of $MnCl_2$ to the extracted particles effectively reconstitutes photoreduction of Q and pheophytin. A low activity of DCIP photoreduction (20 μequiv. per mg Chl/h) was also restored by Mn^{2+}. Pakham and Barber [6] showed that EGTA (EDTA)-washing of the inside-out thylakoid vesicles results in a partial inhibition of DCIP photoreduction and that addition of $MnCl_2$ to the washed particles enhances the activity. The enhancement is, however, not specific to Mn^{2+}, Mg^{2+} and Ca^{2+} being equally effective [6]. In both cases, no data were presented indicating that the enhanced DCIP photoreduction is associated with a stoichiometric increase in the oxygen evolution. Thus, the present work provides clear evidence for the reconstitution of the water oxidation in a Mn-deficient Photosystem II preparation by Mn^{2+} for the first time.

The reactivation of oxygen evolution by Mn^{2+} was, however, incomplete in the particles which had been totally inactivated with 1 mM EDTA, indicating that the removal of Mn is not the sole cause of the inactivation. In this respect of special interest is the observation that the addition of $CaCl_2$ gives rise to a considerable reactivation of oxygen evolution, provided that the EDTA-treated particles are incubated with $CaCl_2$ for 5 min prior to measurements. This strongly suggests that Ca also is an essential component of oxygen-evolving system and that the inactivation caused by the EDTA treatment is partially due to the extraction of Ca^{2+} from the particles.

The involvement of Ca^{2+} in Photosystem II electron transport has been suggested from several lines of evidence [7–14], but its role in situ is not yet known. The present work clearly shows that Ca^{2+} reactivates oxygen evolution of the EDTA-treated particles through a mechanism distinctively different from that of Mn^{2+}. Characteristically, the reactivation proceeds slowly during the incubation of the EDTA-treated particles with Ca^{2+}. This suggests (1) that the binding site of Ca^{2+} is covered by a barrier through which Ca^{2+} can penetrate only slow, (2) that Ca^{2+} causes reassociation of polypeptide(s) that is released by the EDTA treatment from the particles, or (3) that the binding of Ca^{2+} induces a slow rearrangement of the particle structure to a functional state. Experiments are in progress to examine these possibilities and the Ca^{2+} contents before and after the EDTA treatment of the particles.

Pistorius and Schmid [13] recently showed that extraction with EDTA of lyophilized and lysozyme-treated cells of *Anacystis nidulans* results in loss of oxygen evolution and that the activity is resorted on addition of both Mn^{2+} and Ca^{2+}. The synergistic effect of Mn^{2+} and Ca^{2+} was also observed in the Photosystem II preparation employed here. This again support the view that the two cations reactivates oxygen evolution by different mechanisms.

The simultaneous addition of Mn^{2+} and Ca^{2+}, each 5 mM, strongly suppressed, however, the Mn^{2+} effect when the activity was determined shortly after the addition of the two cations, and the Ca^{2+} effect when assayed after 5 min of the incubation. Clearly, the two divalent cations can compete with each other for both Ca^{2+}- and Mn^{2+}-binding sites in the EDTA-treated particles. It appears, therefore, that the two binding sites have low specificity for divalent cations. A competitive relationship between Mn and Ca in the photoactivation of oxygen evolution has been re-

ported in intact chloroplasts isolated from wheat which had been grown under intermittent illumination [12].

The observation that the maximum extent of reactivation is attained only at 5 mM of Mn^{2+} and Ca^{2+} implies that the divalent cations bind loosely to their functional sites in the EDTA-treated particles. Nevertheless, the high levels of the oxygen-evolution activity restored are evidence that Mn^{2+} and Ca^{2+} function in the reconstituted system essentially similarly as the cations do in the untreated particles. Thus, the EDTA-treated particles would serve as a very useful material for the investigation of the roles of Mn^{2+} and Ca^{2+} in the oxygen-evolving system of photosynthesis.

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