Effect of Mn^{2+} and Ca^{2+} on O_2 evolution and on the variable fluorescence yield associated with Photosystem II in preparations of *Anacystis nidulans*

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Extraction with EDTA of lyophilized and lysozyme treated preparations of the blue-green algae *Anacystis nidulans* resulted in loss of the capacity for photoevolution of O_2. Reactivation was achieved by the addition of both cations: Mn^{2+} and Ca^{2+} (or to a smaller extent by Mn^{2+} and Sr^{2+}). The dual requirement for Mn^{2+} and Ca^{2+} could be demonstrated when the O_2 evolution under short saturating light flashes and the variable chlorophyll fluorescence associated with the reduction of the primary acceptor of Photosystem II was examined. The fluorescence experiments in addition showed that incorporation of the cations was a light dependent step, since the fluorescence rise only started after a lag period.

Cyanobacteria Anacystis nidulans *Photosystem II* *Mn^{2+} and Ca^{2+} activation* *O_2 evolution* *Variable fluorescence*

1. INTRODUCTION

We had previously shown that extraction with EDTA of French press particles from the blue-green algae *Anacystis nidulans* resulted in the loss of the O_2 evolving capacity and that both cations Mn^{2+} and Ca^{2+} are required for the reactivation of the O_2 evolution in the EDTA-extracted French press particles [1]. A dual requirement of Mn^{2+} and Ca^{2+} for the activation of the 'latent water-oxidation center' has also been reported for intact chloroplasts isolated from wheat leaves grown under intermittent flash illumination in [2]. A requirement of Ca^{2+} for O_2 evolution and an effect of Ca^{2+} on the chlorophyll fluorescence in Ca^{2+}-depleted cells of *A. nidulans* has recently been shown [3]. Moreover, authors in [4] have reported that Ca^{2+} restores the O_2 evolution activity in salt-washed PS II membranes from spinach where the 23 and 17 kDa polypeptides were depleted. Here we extend our studies with Mn^{2+} and Ca^{2+} in *A. nidulans* to show the effect of both cations on the O_2 evolution pattern under short light flashes and on the variable chlorophyll fluorescence associated with the reduction of the primary acceptor of PS II.

2. MATERIALS AND METHODS

Anacystis nidulans (*Synochococcus leopoliensis*) B 1402-1 was obtained from the Sammlung von Algenkulturen (Universität Göttingen). The growth of cells was the same as in [5]. Lysozyme from egg white (22000 units/mg), RNase from bovine pancreas (40 units/mg) and DNase from bovine pancreas (1000 units/mg) were purchased from Boehringer, Mannheim.

2.1. *Preparation of lyophilized and lysozyme treated cells of* A. nidulans

The lyophilized and lysozyme treated cells of *A. nidulans* were basically prepared as in [6]. The Anacystis cells were harvested after 2 days (cell density about 2 μl cells/ml, total vol. 1500 μl cells) by centrifugation for 15 min at 3000 × *g*, washed once with about 200 ml distilled water and

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resuspended in 15 ml of 0.02 M Hepes buffer (pH 7), containing 5% sucrose and 40 mM $MgCl_2$ (100 μl cells/ml). The sample was lyophilized in two flasks for about 2 h and then resuspended in 15 ml of 0.02 M Hepes buffer (pH 7) containing 40 mM $MgCl_2$. In addition, 90 mg lysozyme (6 mg lysozyme/ml suspension) and 15 μg DNase and 150 μg RNase were added. The sample was kept at 25°C for 90 min in the dark with shaking and was then centrifuged for 10 min at 7700 × g. All subsequent preparation steps were performed at 0–5°C. After the centrifugation the sample was washed with the following solutions: once with 30 ml of 0.02 M Hepes buffer (pH 7) containing 40 mM $MgCl_2$, twice with 30 ml of 0.02 M Hepes buffer (pH 7) containing 5% sucrose and 10 mM EDTA, and once with 30 ml of 0.02 M Hepes buffer (pH 7) containing 5% sucrose. The sample was resuspended in 0.02 M Hepes buffer (pH 7) containing 5% sucrose to give a chlorophyll content of about 0.5 mg/ml. When the sample was washed with EDTA a 10 min incubation period was allowed before recentrifugation.

2.2. *Measurements of O_2 evolution*

O_2 evolution was measured with a Gilson oxygraph (model IC-OXY) fitted with a Clark type electrode as in [7]. The reaction mixture contained in a total volume of 1.85 ml: 100 μmol Mes–Tricine buffer (pH 7.3), 3 μmol ferricyanide, EDTA-washed Anacystis preparation containing 10–20 μg chlorophyll and the cations as indicated in the legend to the table.

O_2 evolution under short light flashes and the O_2 gush was measured with the 3 electrode system described in [8]. Flashes of white light were provided by a Xenon lamp (Stroboscope 1539 A from General Radio). The flash duration was 8 μs. Usually a sequence of 30 flashes was given, spaced 300 ms apart. The O_2 gush was measured on the same electrode, but using red light provided by a halogen lamp (24 V, 250 W) and a red plexiglass filter. The time of illumination was 30 s. The electrode was coated with a very thin layer of collodion for all measurements.

The reaction mixture contained, in a total volume of 1.85 ml, 100 μmol Mes–Tricine buffer (pH 7.3) and the Anacystis preparation containing 100–120 μg chlorophyll. No electron acceptor was provided; 0.5 ml of this mixture was used on the electrode. The first sequence was measured after 20 min to allow sedimentation of the particles on the electrode and dark adaptation. Metal ions were added as indicated in the figure legends. After each addition 5 min were allowed for dark adaptation.

2.3. *Fluorescence measurements*

Fluorescence measurements were performed as in [9]. Excitation was achieved by light filtered through a blue filter (BG 38 from Schott, Mainz) and light emission was detected via a photomultiplier (PM: EMI 9558 QB) after passing the light through a Corning CS 264 filter and a Wratten 97 filter. The reaction mixture was the same as that used for the measurement of the O_2 evolution under short light flashes. One ml of that reaction mixture was filled in a 1-mm cuvette and the dark adaptation time was 5 min.

3. RESULTS

The fast electrode system described in [8] was used to measure O_2 evolution as a consequence of short saturating light flashes but it was necessary to coat the electrode with a very thin membrane of collodion since the Anacystis particles otherwise became inactivated on the electrode. We assume that interaction of the particles with the silver ring of the electrode caused this inactivation, since it has been shown that PS II is sensitive to transition metals, as, e.g., Zn^{2+} [7,10]. The French press particles which we previously prepared from *A. nidulans* [7] gave a rather low yield of O_2 under flash illumination and were thus not very suited for such experiments. We tried other preparations and found that the lyophilized and lysozyme treated Anacystis cells gave a good initial O_2 evolution during flash illumination. Cations could also be extracted from these particles by EDTA and a dependence of the O_2 evolution on added Mn^{2+} and Ca^{2+} could be demonstrated. Since the degree of Mn^{2+} removal from these particles was variable and not quite as effective as shown for the French press particles [1], we give results of two representative experiments in table 1.

3.1. *Effect of Mn^{2+} and Ca^{2+} on the O_2 evolution pattern*

We examined the effect of Mn^{2+} and Ca^{2+} on the O_2 evolution pattern under short saturating

Table 1

Effect of Ca^{2+} and Mn^{2+} on O_2 evolution

Additions	O_2 evolution (μmol $O_2 \cdot$ mg chlorophyll$^{-1} \cdot h^{-1}$)	
	Experiment 1	Experiment 2
None	5.9	0
Ca^{2+}	35.3	19.2
Mn^{2+}	14.5	0
Ca^{2+} and Mn^{2+}	55.9	80.0

Lyophilized and lysozyme treated Anacystis cells were washed twice with EDTA as described in section 2. O_2 evolution in the light with ferricyanide as acceptor was measured as described in section 2. Metal ions were added as indicated. The $CaCl_2$ concentration was 54 mM and the $MnCl_2$ concentration was 0.1 mM. These concentrations were saturating, as has been shown [1]

light flashes to see whether the dual requirement for Mn^{2+} and Ca^{2+} could be demonstrated under these conditions. The EDTA-washed Anacystis preparation without added cations (no ferricyanide added) showed a low O_2 evolution capacity which could be stimulated by addition of Mn^{2+} and Ca^{2+} (fig.1A). Both cations were required to obtain optimal O_2 evolution activity. There is no significant difference in the pattern of O_2 evolution activity – only the amount of O_2 evolved is increased upon addition of Mn^{2+} and Ca^{2+}. The stimulatory effect could also be observed on the O_2 gush (fig.1B). Ca^{2+} alone will give some stimulation of the O_2 evolution since the Mn^{2+} was not totally removed in this preparation. The corresponding values for the stimulation of the O_2 evolution by these cations, when the activity was measured with a Clark-type electrode, is given in table 1, experiment 1.

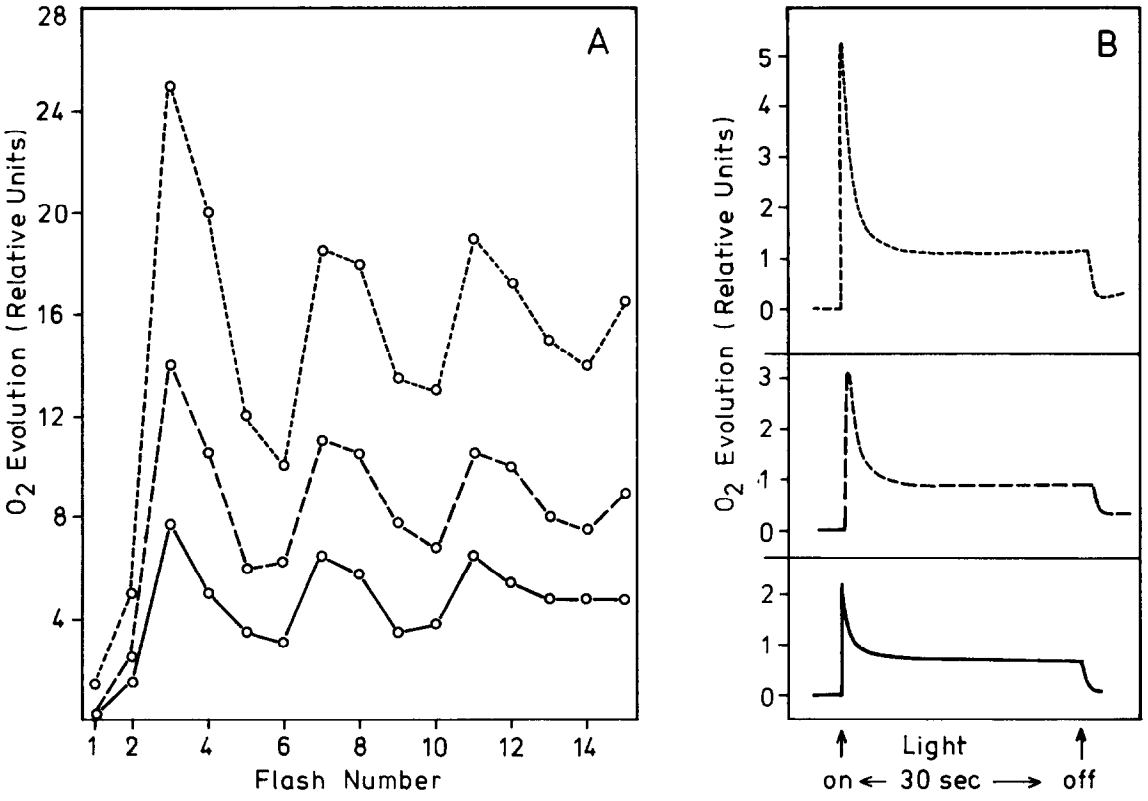

Fig.1. Effect of Mn^{2+} and Ca^{2+} on the O_2 yield under short light flashes and on the O_2 gush. The EDTA-washed Anacystis preparation was suspended in Mes–Tricine buffer (pH 7.3) as described in section 2. No electron acceptor was added. (A) Effect of cations on O_2 yield under short light flashes. (B) Effect of cations on the O_2 gush. (——) O_2 yield in absence of added cations, (– – –) O_2 yield in presence of 54 mM $CaCl_2$, (- - -) O_2 yield in presence of 54 mM $CaCl_2$ and 0.1 mM $MnCl_2$.

3.2. *Effect of cations on the variable fluorescence*

The effect of various cations on the variable chlorophyll fluorescence related to the reduction of the primary electron acceptor of PS II is given in fig.2. In the absence of added cations practically no increase in variable fluorescence (above F_o) was observed. The same result was obtained when Mn^{2+} and Ca^{2+} were added alone. However, addition of Mn^{2+} and Ca^{2+} together resulted in a substantial increase in the variable fluorescence yield. This increase only started after a lag period of about 200 ms and then steadily rose till the maximum was reached. When a dark period of about 2 min was allowed, the fluorescence fell back to F_o and light activation could be observed again. The second highest rise in variable fluorescence was observed with the combination of Mn^{2+} and Sr^{2+} while all other combinations tested (Mn^{2+} and Mg^{2+}, Mn^{2+} and Ba^{2+} or Co^{2+} and Ca^{2+}, not shown) were ineffective.

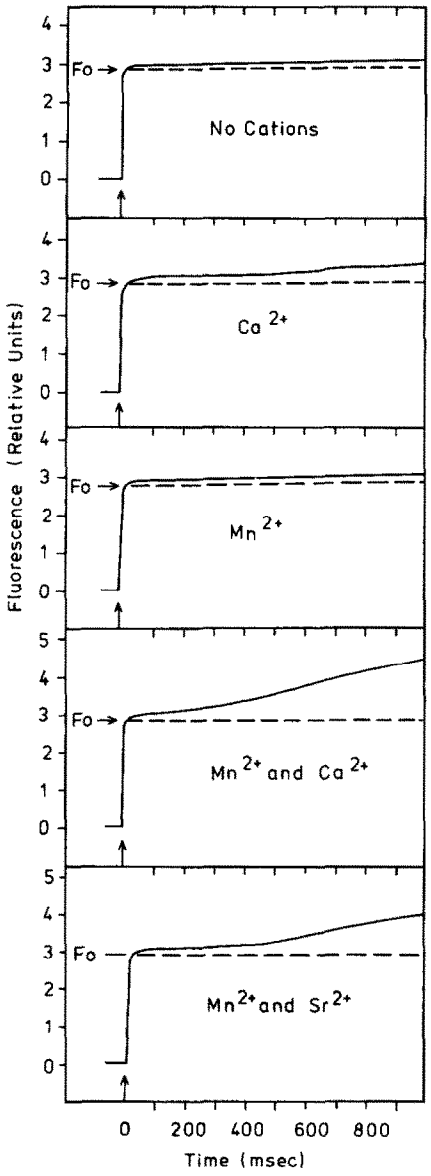

Fig.2. Effect of cations on chlorophyll fluorescence. The EDTA-washed Anacystis preparation was suspended in Mes–Tricine buffer (pH 7.3) as described in section 2 and cations were added as indicated in the figure. The $CaCl_2$ and $SrCl_2$ concentration was 54 mM and the $MnCl_2$ concentration was 0.1 mM. The arrow indicated the onset of illumination.

The corresponding values for the O_2 evolution measured with a Clark electrode are given in table 1, experiment 2. These results obtained with various cations on the variable fluorescence are in full agreement with our previous results which showed that only the combination of Mn^{2+} and Ca^{2+} (and Mn^{2+} and Sr^{2+}) resulted in an activation of the O_2 evolution in EDTA-extracted French press particles of *A. nidulans* [1].

4. DISCUSSION

The dual requirement for Mn^{2+} and Ca^{2+} can be demonstrated for the O_2 evolution under short light flashes as well as for the variable chlorophyll fluorescence associated with PS II. This further supports the conclusion that both cations are required for the early reactions of the PS II reaction center. In the absence of Mn^{2+} and Ca^{2+} electron transport through PS II appears to be interrupted at the donor side of PS II and this results in a condition where 'Q' prevails in the oxidized form with low fluorescence. The fluorescence experiments in addition show that full restoration by Mn^{2+} and Ca^{2+} of the electron flow from H_2O to the primary acceptor of PS II is a light-requiring step. The requirement of light for Mn^{2+} incorporation is well documented and it is believed that one of the higher oxidation states of Mn (Mn^{3+} or Mn^{4+}) is the active form [2,11]. Our experiments show that this light activation only proceeds when Ca^{2+} (or Sr^{2+}) is present together with Mn^{2+}.

The relatively easy extraction of the essential Mn^{2+} from Anacystis preparations by EDTA is quite unusual. Although a number of treatments

have been reported for the removal of Mn^{2+} from chloroplasts, e.g., incubation with Tris buffer, hydroxylamine or high concentrations of certain cations, and heating, washing with EDTA was not effective [12]. Even treatment of PS II particles from plants with EDTA resulted in removal of only a very small fraction of the essential Mn^{2+} [13]. The only described preparation from eukaryotes, to our knowledge, that allows Mn extraction by EDTA are inside-out vesicles prepared from pea chloroplasts [14]. It is well documented that blue-green algae have a more loosely organized thylakoid membrane than plants [15] and this could be the reason for the relatively easy extraction of the essential Mn from Anacystis preparations. Moreover, the frequently observed 'over-reduced' state of the PS II reaction center that we see when we study the O_2 evolution pattern under short light flashes (unpublished) might contribute to this, since authors in [16] have recently reported that reducing conditions favor the release of Mn from thylakoid preparations.

Our experiments show very clearly the dual requirement of PS II in *A. nidulans* for Mn^{2+} and Ca^{2+}. However, it is unclear to what protein(s) the two cations are bound in the reaction center of PS II. Evidence seems to accumulate that Mn^{2+} is possibly bound to the 34-kDa polypeptide of PS II [16,17] and it has been suggested that Ca^{2+} might be bound through the 23-kDa polypeptide. However, these experiments also showed that Ca^{2+} can restore O_2 evolution in the absence of the 23-kDa polypeptide [4]. This indicates that Ca^{2+} can directly, and possibly also through the 23-kDa polypeptide, regulate the PS II activity.

We had previously shown that a flavin protein with L-amino acid oxidase is associated with PS II of *A. nidulans* and that the amino acid oxidase activity is inhibited by Ca^{2+} to about the same extent as the photosynthetic O_2 evolution is stimulated by Ca^{2+}. Therefore, an involvement of this flavin protein in the redox reactions of PS II in *A. nidulans* has been suggested [1,7]. Moreover, our previous experiments with the isolated flavin protein have shown that Ca^{2+} can alter the spectrum of the oxidized flavin in the enzyme to a form which is similar to the semiquinone form of the flavin [1]. Since ESR experiments have indicted that 'Signal II' associated with PS II is due to a metal-quinone complex and since it was suggested [18] that the metal is not a transition metal but a diamagnetic cation, like Ca^{2+}, a connection to our experiments is quite possible. Further experiments about the role of the flavin protein and its possible involvement in the cation binding are in progress.

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