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A simple procedure to determine Ca^{2+} in oxygen-evolving preparations from *Synechococcus* sp.

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A simple procedure to determine Ca^{2+} bound to low- and high-affinity sites of *Synechococcus* oxygen-evolving particles was developed. The method consists of determination of Ca^{2+} in the particle suspensions with and without treatment with a chelating resin, Chelex 100, to remove the metal cations contaminating the suspension medium as well as those weakly bound to the particles. It was found that the particles contain one tightly bound Ca^{2+} per PS II reaction center which cannot be extracted with Chelex 100 and a larger amount of weakly associated and resin-extractable Ca^{2+}.

Ca^{2+} *Chelex 100* *Oxygen-evolving particle* *Photosystem II* (Synechococcus)

1. INTRODUCTION

During the last several years, evidence has accumulated indicating that calcium functions in photosynthetic electron transport related to PS II in higher plants and cyanobacteria (reviews [1,2]). Ca^{2+} stimulates photoactivation of oxygen evolution in several chloroplast systems [3–5], and calmodulin antagonists inhibit PS II electron transport [6,7]. Oxygen evolution of PS II membrane preparations inactivated by washing with high concentrations of NaCl or $CaCl_2$ is considerably reactivated by addition of $CaCl_2$ [8–11]. Requirement of Ca^{2+} for PS II electron transport in cyanobacteria has been demonstrated simply by depleting the metal cations from cells, thylakoid membranes or PS II preparations: The depletion blocks oxygen evolution but high rates of oxygen evolution are recovered on addition of Ca^{2+} to the deficient cells or by preparing the thylakoid membranes and PS II particles in the presence of the metal cations [12–14]. *Synechococcus* oxygen-evolving preparations were inactivated by treatment with EDTA in a hypotonic medium and the lost activity was partially restored after incubation with 5 mM $CaCl_2$ [15]. Fluorescence measurements and other experiments led Brand et al. [16] to the conclusion that the site of action of Ca^{2+} is at the PS II reaction center, or immediately to its oxidizing side. More recently, the functional site of Ca^{2+} was identified as electron transport between P680 and Z [17].

It is essential to determine the number of calcium atoms associated with the PS II reaction center for the understanding of the function of Ca^{2+} in photosynthetic electron transport. However, accurate determination of Ca^{2+} in biological materials is not so simple because calcium is notorious for contaminating various chemicals and glasswares and in addition proteins and other organic compounds more or less have affinity for Ca^{2+}. Only two and markedly different values have been reported for the abundance of Ca^{2+} in oxygen-evolving PS II preparations [9,18].

Here we report a simple method to determine

Abbreviations: PS, photosystem; Mes, 2-(*N*-morpholino)ethanesulfonic acid

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Ca^{2+} in *Synechococcus* oxygen-evolving particles. The method employs a cation-chelating resin, Chelex 100, to remove not only Ca^{2+} contaminating the suspending medium but also the metal cations loosely bound to the particles, and thus shortens laborious pretreatments of chemicals, wares and samples to eliminate contaminating Ca^{2+}. The results show that the oxygen-evolving preparations contain one tightly bound Ca^{2+} per PS II reaction center.

2. MATERIALS AND METHODS

Oxygen-evolving PS II particles were isolated from *Synechococcus* thylakoid membranes with β-octylglucoside as in [15]. The preparations were suspended in 40 mM Mes-NaOH (pH 5.7), 10 mM NaCl, 1 mM $MgCl_2$ and 0.5 M sucrose. Ca^{2+} was determined with a Shimadzu atomic absorption spectrophotometer (AA640-01) equipped with a graphite furnace atomizer (GFA-2). To remove contaminating Ca^{2+} from samples and suspending media, 0.1–0.3 Chelex 100 (100–200 mesh, Bio-Rad) was added to 0.5 ml of the particle suspension. The suspension was shaken at a rate of one stroke per 10 s for 1 min, then kept still for 2 min to sediment the resins. The supernatant was assayed for Ca^{2+} after dilution with 2 vols water. Cycles of the 1-min treatment with Chelex 100 and the 2-min standing to sediment the resins were repeated and Ca^{2+} was determined every 3 min until a constant level of Ca^{2+} was attained.

Water was deionized, distilled, and stored in a plastic bottle, and further treated with Chelex 100 immediately prior to use. Otherwise, no special attempts were made to eliminate Ca^{2+} from chemicals and glass and plastic wares used. Chelex 100 was suspended in water, brought to pH 6.5 with HCl, washed extensively with water and dried. The addition of Chelex 100 shifted the pH of the sample suspension from 5.7 to about 6.3.

Oxygen evolution was measured at 40°C with a Clark-type oxygen electrode under illumination with saturating white light [15]. The reaction mixture contained 1 M sucrose, 50 mM Mes-NaOH (pH 5.5), 10 mM NaCl, 5 mM $MgCl_2$, 1 mM ferricyanide and 0.5% digitonin. Chlorophyll *a* was determined by the method of Mackinney [19].

3. RESULTS

Fig.1 shows concentrations of Ca^{2+} in the media containing various amounts of the oxygen-evolving particles, which were measured without any attempts to eliminate contaminating Ca^{2+} from the suspending media and the particles. The suspending medium contained considerable and variable concentrations of Ca^{2+} and, in experiments shown in fig.1, Ca^{2+} present in the medium amounted to 26 μM. The concentration of Ca^{2+} increased linearly with increasing amounts of the particles added and the abundance of Ca^{2+} associated with the particles was estimated as about 10 Ca^{2+} per 48 chlorophyll *a*. Because the oxygen-evolving particles have one Q_A per 48 chlorophyll *a* [15], the amount of the bound Ca^{2+} corresponds to about 10 Ca^{2+} per PS II reaction center.

The bound Ca^{2+} thus determined varied significantly with preparations. An attempt was made to remove loosely bound Ca^{2+} with EDTA only to find that the chelator itself was contaminated with a significant amount of Ca^{2+}. The loosely bound Ca^{2+} was found to be removed effectively with a cation-chelating resin, Chelex 100.

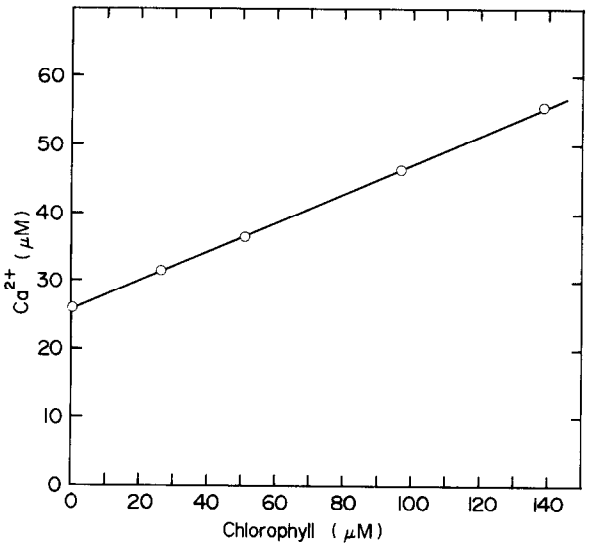

Fig.1. Ca^{2+} concentrations of media containing various amounts of oxygen-evolving particles. No attempts to remove contaminating Ca^{2+} from the suspending media or particles were made and Ca^{2+} was determined directly with the suspensions.

Fig.2 shows changes in the Ca^{2+} concentration of the particle suspensions which was determined after each cycle of the 1-min shaking with Chelex 100 and the 2-min standing to precipitate the resins as described in section 2. The initial concentrations of Ca^{2+} in the suspensions exceeded 20 μM (not shown in fig.2). Chelex 100 removed Ca^{2+} present in the media rapidly and thoroughly; the Ca^{2+} concentration became negligible after one or two cycles of Chelex treatment in the absence of the particles (curve a). Ca^{2+} decreased more slowly in the presence of the particles, probably reflecting a slow equilibration of Ca^{2+} between low-affinity sites and the medium or the resins. In addition, Chelex 100 could not extract all the metal cations associated with the particles. Note that the constant levels of Ca^{2+} attained after 5 or 6 cycles of the Chelex 100 treatment were proportional to the amounts of the particles added, all giving ratios of about 1 Ca^{2+} per 48 chlorophyll *a* (curves b–d). The results indicate that most of bound Ca^{2+} are associated with low-affinity sites but a small amount of Ca^{2+}, which is in stoichiometry to the PS II reaction center, binds strongly to the particles.

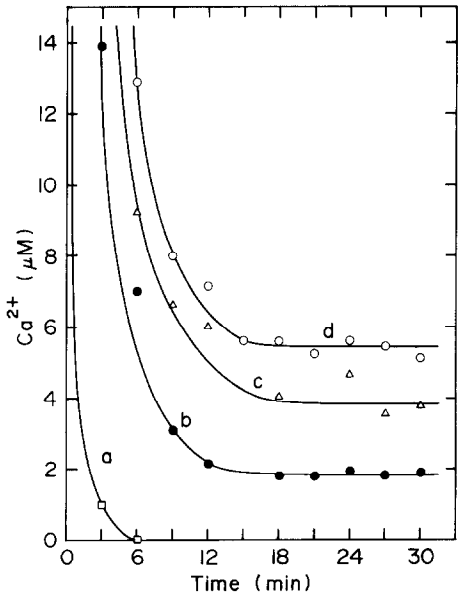

Fig.2. Effects of Chelex 100 treatment on Ca^{2+} concentrations of media containing various amounts of oxygen-evolving particles. Chelex 100 treatment was carried out as described in section 2. Curves: a, no oxygen-evolving particles; b, c and d, particles containing 90, 177 and 253 μM chlorophyll *a*, respectively, were added.

The optimum concentration of sucrose for oxygen evolution in *Synechococcus* particles is 1 M [15]. However, such a high concentration of sucrose interfered with atomic absorption spectrometry and it was necessary to dilute the suspension with 4 or 5 vols water prior to measurement. Because the ratios of the tightly bound Ca^{2+} to PS II did not vary significantly between 0.5 and 1 M sucrose (not shown), experiments shown in fig.2 were carried out in the presence of 0.5 M sucrose. It should be stressed that rates of oxygen evolution were not appreciably affected by the Chelex treatment in the presence of 0.5 M sucrose, provided that the activity was measured in medium containing 1 M sucrose (not shown). However, the treatment at sucrose concentrations below 0.5 M caused gradual decreases both in amount of the tightly bound Ca^{2+} and in rate of oxygen evolution.

It is important to keep the pH of the sample suspension below 7.0 during the Chelex treatment. The amount of tightly bound Ca^{2+} per PS II reaction center was consistently 1 between pH 5.5 and 7.0 but decreased to 0.6 to 0.7 at higher pH values (not shown). Caution is needed in that the addition of Chelex 100 (sodium form) causes a considerable alkalinization of the medium pH, unless the resin is adjusted to an appropriate pH prior to use.

4. DISCUSSION

This work demonstrates that *Synechococcus* oxygen-evolving particles are associated with one tightly bound Ca^{2+} and larger amounts of loosely bound Ca^{2+}. The washing of the particles with EDTA to extract exogenous Ca^{2+} yielded ambiguous results because EDTA itself is contaminated with Ca^{2+} and removal of EDTA by washing or dialysis newly introduced contamination of the metal cations. An obvious advantage of Chelex 100 over EDTA is that the resins can be readily separated from the sample suspension by sedimentation, enabling us to determine Ca^{2+} directly with the supernatant. An important consequence of the method is that exogenous Ca^{2+} present in the suspending medium is also eliminated by the Chelex 100 treatment. Thus, the method allows determination of Ca^{2+} without laborious

pretreatments of chemicals and glasswares to make them Ca^{2+}-free.

By virtue of its simplicity, we expect that the method developed here would have a wider application to various biological materials. In this respect, the following points are worthy of mention: (i) Chelex 100 is a rather weak chelating reagent, having an apparent stability constant of 4.5×10^2 at pH 6.7 for its Ca^{2+} complex [20]. Thus, the resin removes only free and weakly bound Ca^{2+}. (ii) Samples should be treated gently with Chelex 100 because vigorous agitation of the particles with the resins often causes significant inactivation. (iii) The time needed to extract all the weakly bound Ca^{2+} can, however, be shortened by increasing amounts of the resin added and by shaking the suspension gently but more frequently. The Ca^{2+} content of the purified oxygen-evolving complexes from the cyanobacterium was determined by adding 0.5 g Chelex 100 to 1.0 ml sample suspension and then by incubating the suspension for 5–10 min with gentle stirring [21].

The involvement of Ca^{2+} in PS II electron transport near P680 in *Synechococcus* PS II particles has been demonstrated [15]. Ca^{2+} bound to low-affinity sites is not related to the activity because the removal of the loosely bound Ca^{2+} has no effect on the rate of oxygen evolution. It is concluded therefore that the tightly bound Ca^{2+}, which is present at a stoichiometric concentration to the PS II reaction center, is important to PS II electron transport.

The above conclusion is at the first sight contradictory to the previous observation that the maximal reactivation of oxygen evolution was attained at 5 mM $CaCl_2$ in *Synechococcus* particles which had been inactivated with EDTA in a hypotonic medium [15]. The requirement of such a high concentration of Ca^{2+} implies that weakly bound Ca^{2+} is responsible for the reactivation. However, EDTA inhibits oxygen evolution only in a medium containing a low concentration of sucrose but not in the presence of 1 M sucrose [15]. This suggests that the Ca^{2+} binding is strongly affected in a hypotonic medium. Thus there would be two explanations; first, the binding of Ca^{2+} to its functional site is weakened by a structural change of the particles induced in a hypotonic environment or, second, the functional site has a low affinity for Ca^{2+} but is structurally shielded from the outer aqueous phase unless the particles are exposed to a hypotonic medium.

There are only two reports on the abundance of Ca^{2+} in other oxygen-evolving membrane preparations. An extremely high value of 0.86 Ca^{2+}/chlorophyll was reported in spinach preparations, but the method employed seems not to distinguish between exogenous and endogenous Ca^{2+} present in the sample suspensions [9]. A lower value of 2 Ca^{2+}/200 chlorophyll bound with high affinity in wheat preparation was mentioned in [18], but details of the result and method were not reported. Experiments to determine the number of calcium atoms associated with PS II preparations from chloroplasts by the Chelex method are in progress.

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