A requirement for Ca^{2+} in the extraction of O_2 -evolving Photosystem 2 preparations from the cyanobacterium *Anacystis nidulans*

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Ca^{2+} has been shown to be essential for the retention of maximal O_2 -evolving activity in Photosystem 2 particles extracted by using dodecyldimethylamine oxide from *Anacystis nidulans* thylakoids. The effect cannot entirely be mimicked by using Mg^{2+} . Ca^{2+} stimulates electron transport from diphenylcarbazine to 2,6-dichloroindophenol catalysed by lead-inhibited cation-free preparations, showing the presence of two cation-binding sites in these particles. Photosystem 2 preparations extracted in Ca^{2+} -containing buffer show the presence of three polypeptides at mol. wt. 30 000, 33 000 and 36 000, which are absent or much decreased in preparations extracted in Mg^{2+} -containing buffer. The calmodulin antagonist chlorpromazine inhibits activity of the Photosystem 2 preparation, suggesting the presence of a Ca^{2+} -binding protein.

Although much progress has been made in the isolation and characterization of Photosystem 1 (e.g. Bengis & Nelson, 1975; Satoh & Butler, 1978), many more difficulties have been encountered in the isolation of Photosystem 2 particles retaining O_2 -evolving capacity. The most active preparations to be reported have been isolated from cyanobacteria by Stewart & Bendall (1981) and ourselves (England & Evans, 1981) and from spinach chloroplasts by Yamamoto *et al.* (1981). Studies on O_2 -evolving cell-free preparations from various cyanobacteria have demonstrated a requirement for Ca^{2+} to give maximal activity (Piccioni & Mauzerall, 1976; Brand, 1979; Yu & Brand, 1980) and recent work by Yerkes & Babcock (1981) suggests that Ca^{2+} may have a role in the rate of re-reduction of the Photosystem 2 reaction-centre chlorophyll (measured as e.p.r. signal II_f) in spinach chloroplasts. Barr *et al.* (1982) have shown that antagonists to the Ca^{2+} -binding protein calmodulin can inhibit Photosystem 2 activity in chloroplasts. Although no requirement for Ca^{2+} was demonstrated by Stewart & Bendall (1981) in the isolation of O_2 -evolving Photosystem 2 particles from the thermophilic cyanobacterium, *Phormidium laminosum*, we do find such a requirement with *Anacystis nidulans*. Here we describe this effect on the activity of the preparations and on their polypeptide composition.

Methods

Anacystis nidulans (Collection of Algae; UTEX 625; University of Texas, Austin, TX, USA) was a gift from Dr. N. G. Carr. The organism was maintained and grown, cell-free membrane preparations extracted and O_2 -evolving Photosystem 2 particles prepared as described previously (England & Evans, 1981). 'Calcium buffer' contained 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 25% (v/v) glycerol and 30 mM- or 50 mM- CaCl_2 as stated in the text. In the 'magnesium buffer', the CaCl_2 was replaced by MgCl_2 . Polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969). Light-induced O_2 evolution was measured in a Rank oxygen electrode attached to an MSE Spectroplus. Chlorophyll *a* was assayed by the method of Strain *et al.* (1971). Reduction of 2,6-dichloroindophenol was measured at 590 nm in a Perkin-Elmer 550 spectrophotometer, the sample being illuminated via a fibre optic by a 100 W tungsten/iodine lamp covered by a Kodak Wrattan 70 gelatine filter, the photomultiplier being covered by a Corning 9592 turquoise filter.

All chemicals were of Analar grade wherever possible, glycerol being purchased from BDH. Dodecyldimethylamine oxide was a gift from Harshaw Chemicals, Glasgow, Scotland, U.K.

Results and discussion

The first effect of Ca^{2+} on preparation of Photosystem particles was noticed at the stage of

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Table 1. *Light-induced O₂-evolution activity of cell-free thylakoids of Anacystis nidulans*

Chlorophyll *a* concentration was 10 µg/ml, with 2 mM-potassium ferricyanide as electron acceptor, in a final volume of 3 ml, containing also 0.5 M-sorbitol, 10 mM-Hepes and 5 mM-phosphate, pH 7.5.

(A) Ca²⁺ or Mg²⁺ added to the cell-breakage medium

Cation concentration	Rate of O ₂ evolution (µmol of O ₂ /mg of chlorophyll <i>a</i> per h)
0 mM-MgCl ₂	31.0
30 mM-MgCl ₂	46.0
50 mM-MgCl ₂	46.0
0 mM-CaCl ₂	31.0
30 mM-CaCl ₂	64.6
50 mM-CaCl ₂	68.4

(B) No cation added for cell breakage; Ca²⁺ or Mg²⁺ added to assay medium.

Cation concentration (mM)	Rate of O ₂ evolution (µmol of O ₂ /mg of chlorophyll <i>a</i> per h)	
	+MgCl ₂	+CaCl ₂
0.0	16	16
2.5	27	18
5.0	35	28
10.0	35	32
20.0	35	35
30.0	35	35

Table 2. *The effects of Ca²⁺ and Mg²⁺ on the O₂-evolving activity of Photosystem 2 particles*

Experimental conditions were as described in the legend to Table 1.

Experimental condition	Light-induced O ₂ evolution (µmol/mg of chlorophyll <i>a</i> per h)
Calcium buffer throughout	694
Magnesium buffer throughout	139
No cation throughout	75
10 mM-CaCl ₂ in gradient only	249
30 mM-CaCl ₂ in gradient only	287

cell breakage, an effect similar to that noted by Brand (1979). This effect is shown in Table 1. Clearly, addition of Ca²⁺ to the cell-breakage medium is more effective in maintaining O₂ evolution activity than Mg²⁺, but subsequent addition of either cation to thylakoids prepared in the absence of cation has a similar increasing effect on activity. Maintaining an optimal cation concentration throughout the thylakoid preparation gives, in a typical experiment, rates of 270 µmol of

O₂/mg of chlorophyll *a* per h for a preparation broken and resuspended in calcium buffer, and 216 µmol of O₂/mg of chlorophyll per h for the similar preparation in magnesium buffer. Thus there would appear to be two effects of Ca²⁺ on thylakoid membranes, one unique to Ca²⁺, which cannot be mimicked by Mg²⁺, requiring Ca²⁺ to be present at the time of cell breakage, and a second effect, a 'cation effect', which can be produced by adding Mg²⁺ or Ca²⁺ to cell-free thylakoid preparations. The preparation method for Photosystem 2 particles (England & Evans, 1981) requires thylakoids to be extracted with the detergent dodecyltrimethylamine oxide and the appropriate fraction isolated from a sucrose density gradient. Table 2 illustrates the difference between particles isolated in magnesium buffer and those in calcium buffer. With magnesium buffer throughout the preparation, the Photosystem 2 particles have less O₂-evolving activity than the original thylakoid membranes, whereas use of the calcium buffer results in an active preparation. It was thought that Ca²⁺ might dispose the thylakoid membranes into a conformation facilitating attack by dodecyltrimethylamine oxide, so the effect of Ca²⁺ addition only at the sucrose-gradient stage of the preparation was assayed. These results are also shown in Table 2, and it is clear that substantial activity can be restored to the preparation after the dodecyltrimethylamine oxide extraction step by subsequent addition of Ca²⁺. Yerkes & Babcock (1981) have suggested that Ca²⁺ binds to Z on the donor side of Photosystem 2 in spinach chloroplasts. The effects of Ca²⁺ on the donation of diphenylcarbonohydrazide to Photosystem 2 in the presence and absence of Pb²⁺ was therefore investigated. Pb²⁺ has been proposed to inhibit Photosystem 2 on the O₂-evolving side (Wong & Govindjee, 1976), and it might be thought that it would do so at a proposed Ca²⁺-binding site. This is not the case, as is shown in Table 3, where Photosystem 2 activity is measured as reduction of 2,6-dichloroindophenol. That Pb²⁺ inhibits electron transport before the site of donation of diphenylcarbonohydrazide in Photosystem 2 particles in a similar fashion to chloroplasts is shown in Table 3(A). We have also been able to repeat the fluorescence data of Wong & Govindjee (1976) for Photosystem 2 particles. Table 3(B) demonstrates that Ca²⁺ can stimulate the rate of electron transport using diphenylcarbonohydrazide as electron donor in Pb²⁺-inhibited particles. This effect is not mimicked by Mg²⁺, and thus places the site of Ca²⁺ action between the Pb²⁺ inhibition site and pigment P680. There are therefore two sites of cation interaction on the O₂-evolving side of Photosystem 2.

Fig. 1 shows the effect of the Ca²⁺-binding protein antagonist, chlorpromazine, on electron transport of Photosystem 2 particles. Chlorpromazine inhibits

Table 3. Photosystem 2 activity of various preparations with water or diphenylcarbonohydrazide as electron donor

Activity was measured as light-induced 2,6-dichloroindophenol reduction. Chlorophyll *a* (10 μ g/ml), 2,6-dichloroindophenol (20 μ M) and diphenylcarbonohydrazide (0.1 mM) were present in a final volume of 2.5 ml also containing 0.5 M-sorbitol, 10 mM-Hepes and 5 μ M-phosphate, pH 7.5.

(A) Photosystem 2 particles prepared using calcium buffer throughout

Treatment	Rate (μ M-2,6-dichloroindophenol/mg of chlorophyll <i>a</i> per h)
No additions	38
+200 μ M-Pb ²⁺	0
+200 μ M-Pb ²⁺ + 0.1 mM-diphenylcarbonohydrazide	50

(B) Photosystem 2 particles prepared using zero cation buffer.

No additions	6
+15 mM-CaCl ₂	6
+15 mM-MgCl ₂	6
No additions	6
+190 μ M-Pb(NO ₃) ₂	0
+190 μ M-Pb(NO ₃) ₂ + 0.1 mM-diphenylcarbonohydrazide	5
+190 μ M-Pb(NO ₃) ₂ + 0.1 mM-diphenylcarbonohydrazide + 15 mM-MgCl ₂	6
+190 μ M-Pb(NO ₃) ₂ + 0.1 mM-diphenylcarbonohydrazide + 15 mM-CaCl ₂	17

Fig. 1. The effect of chlorpromazine on electron transport catalysed by Photosystem 2 particles

●, Particles prepared with calcium buffer; ○, particles prepared with magnesium buffer. Experimental conditions were as described in the legend to Table 3.

Fig. 2. Polyacrylamide-gel-electrophoresis scans of Photosystem 2 particles prepared in (a) magnesium buffer and (b) calcium buffer

Molecular weight of peaks are marked as $10^{-3} \times$ mol.wt., determined by comparison with bovine serum albumin, ovalbumin and cytochrome *c* (not shown).

light-induced 2,6-dichloroindophenol reduction in Photosystem 2 particles prepared in calcium buffer by 94%, with a half-inhibitory concentration of 130 μ M. At higher concentrations chlorpromazine is also seen to inhibit electron transport by Photo-

system 2 particles prepared in magnesium buffer by 55%, with a half-inhibitory concentration of $250\mu\text{M}$. Thus chlorpromazine has a major inhibitory effect at low concentrations on Photosystem 2 prepared in the presence of calcium, but clearly has a secondary inhibitory effect on Photosystem 2 electron transport at higher concentrations manifest in the lesser inhibition of the Photosystem 2 particles prepared in the presence of Mg^{2+} . The concentration of chlorpromazine found to be necessary to inhibit 50% of Photosystem 2 electron transport in spinach chloroplasts by Barr *et al.* (1982) was $150\mu\text{M}$, comparable with the concentration required using Photosystem 2 particles prepared in the presence of Ca^{2+} .

The effect of Ca^{2+} on the polypeptide composition of Photosystem 2 particles is shown in Fig. 2. This shows typical polyacrylamide-gel-electrophoresis patterns for Photosystem 2 particles prepared in calcium buffer and in magnesium buffer. The identity of various polypeptide components in these gels is not discussed herein, but the effect of Ca^{2+} can be pointed out. The major differences between the gel patterns are in the 30000–40000-mol.wt. regions. Bands at mol.wt. 30000 and 36000 are substantially decreased and a band at mol.wt. 33000 absent from Photosystem 2 particles prepared in the absence of Ca^{2+} . There is a small increase in these gels in the proportion of the higher-molecular-weight species at mol.wt. 77000 and 60000. Thus from the calmodulin inhibitor experiments described it seems possible that there is a Ca^{2+} -binding protein within the Photosystem 2 particles. The polyacrylamide-gel-electrophoresis analysis may indicate that Ca^{2+} is somehow responsible for retaining certain peptides within the particles.

Yamamoto *et al.* (1981) found that three polypeptides were removed by Tris washing of O_2 -evolving Photosystem 2 particles from spinach chloroplasts. These had molecular weights of 33000, 24000 and 18000. It would be interesting to speculate that the mol.wt. 33000 polypeptide that

decreased in the absence of Ca^{2+} was the same as that removed by Tris washing or that lost in algal and maize mutants with deletions in Photosystem 2 (Metz *et al.*, 1980; Leto & Miles, 1980).

In conclusion, we find that the presence of Ca^{2+} in the buffer system is essential to the retention of maximal O_2 -evolving activity in Photosystem 2 particles. This level of activity appears to be associated with the retention of polypeptides of mol.wt. 30000, 33000 and 36000 within the particles. The binding site of Ca^{2+} appears to be on the O_2 -evolving side of Photosystem 2, as already proposed by Yerkes & Babcock (1981), and is distinct from the binding site of Pb^{2+} .

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