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CALCIUM AND PHOTOSYNTHETIC OXYGEN EVOLUTION IN CYANOBACTERIA

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Summary

Calcium activation of oxygen evolution from French-press preparations of *Phormidium luridum* is largely reversible upon removal of added Ca^{2+}. Activation occurs via a first-order binding with a dissociation constant of 2.8 mM. An 8-fold increase in oxygen evolution rate observed upon Ca^{2+} addition is accounted for by a 4-fold increase in the number of active photosynthetic units, and a doubling of turnover rate. While both Ca^{2+} and Mg^{2+} stimulate turnover, unit activation is Ca^{2+} specific. Under optimal conditions, 30% of the units functioning in the intact cell can be recovered in the Ca^{2+}-activated preparation.

The Ca^{2+} requirement of *P. luridum* preparations is not relieved by proton-carrying uncouplers, or by rate-saturating concentrations of the Hill acceptor, ferricyanide. Taken together with the reported stimulation by Ca^{2+} of oxygen evolution in the presence of DCMU (Piccioni, R.G. and Mauzerall, D.C. (1976) Biochim. Biophys. Acta 423, 605—609) these observations strongly suggest a site of Ca^{2+} action within Photosystem II.

The pronounced specificity of the Ca^{2+} requirement appears in preparations of other cyanobacteria (*Anabaena flos-aquae* and *Anacystis nidulans*) but not in the eucaryote *Chlorella vulgaris*. While milder cell-disruption methods bring about some Ca^{2+} dependence in *P. luridum*, French-press treatment is required for maximal expression of Ca^{2+}-specific effects. French-press breakage causes a release of endogenous Ca^{2+} from cells, supporting the view that added Ca^{2+} restores oxygen evolution by satisfying a physiological requirement for the cation.

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Abbreviations: CCCP, carbonylcyanide 3-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

Introduction

French-press preparations of the cyanobacterium *Phormidium luridum* show marked stimulation of oxygen evolution activity upon addition of Ca^{2+}, maximal Hill reaction rates obtaining at approx. 10^{-2} M $CaCl_2$ [1]. Comparable concentrations of $MgCl_2$ have only a small stimulatory effect. The Ca^{2+} requirement is persistent as well as specific; Ca^{2+} stimulation of oxygen evolution activity is not diminished by the addition of bovine serum albumin, which binds potentially interfering fatty acids, or by the presence of DCMU. The latter inhibitor lowers but does not eliminate oxygen evolution from *P. luridum* preparations [1,2]; stimulation by Ca^{2+} of the DCMU-insensitive component of oxygen evolution activity suggests that Ca^{2+} acts within the Photosystem II reaction center or water-oxidation apparatus [1].

The character and scope of the effects of Ca^{2+} on oxygen evolution have now been more thoroughly investigated. We report here results pertaining to the physiological significance of the observed Ca^{2+} dependence, its phenomenological description in terms of photosynthetic unit activation and turnover facilitation, and its expression in preparations of varying structural integrity and species. We conclude that Ca^{2+}, crucial to the operation of the cyanobacterial Photosystem II in vivo, is released from thylakoids after French-press treatment. An accompanying report [3] will examine the redox-potential sensitivity and Hill acceptor requirements of Ca^{2+}-dependent *P. luridum* preparations.

Materials and Methods

Culture of algae. P. luridum var. *olivacae* Indiana Culture Collection (I.C.C.) No. 426, *Anabaena flos-aquae*, I.C.C. No. 144, and *Anacistis nidulans*, I.C.C. No. 625, were grown at 37°C in a modified form of Kratz and Myers liquid medium 'D' [4]. EDTA and $Fe_2(SO_4)_3$ additions specified by these authors were omitted, and replaced with Na_2EDTA (114 mg/l) and NaFe(III)EDTA (Calbiochem, 6.5 mg/l). The modifications were made to prevent precipitation of medium components during autoclaving, and to insure an adequate supply of utilizable Fe. Cells were grown at 37°C under $1 \cdot 10^4$—$2 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} fluorescent light and a purged atmosphere of 1% CO_2 in air. 0.5-l flasks containing 200 ml medium were secured to a mechanical shaker oscillating at about 2 Hz. Doubling times in log phase were 8—12 h.

French-press preparations. Unless otherwise indicated, the procedure used in the isolation of French-press preparations was as follows: cells from an actively growing culture were harvested by centrifugation, and washed twice with buffer (10 mM MES, 5 mM NaOH, 30 mM NaCl, pH 6.2) containing 1—5 mM EGTA. After washing in buffer free of EGTA, cells were resuspended at 75—150 μg chlorophyll per ml of ice-cold, NaCl-free buffer containing 8 mg 'Pentex' defatted bovine serum albumin (Miles Laboratories 'Fraction V') per ml, found to stabilize activity [1]. The suspension was passed once, at 5°C and a pressure of 24 000 lb/inch^2 through a French-press fitted with a Teflon ball valve. The effluent was layered over 0.4 M sucrose, NaCl-free MES buffer, and centrifuged at 2000 $\times g$, 5°C for 10 min. In a dark coldroom, aliquots of the

supernatant were added to vials and stored in liquid nitrogen. The yield of chlorophyll in the supernatant was about 95% of the total present in the washed cell suspension. Whole cell contamination, determined by microscopic examination, was less than 1% on a chlorophyll basis.

Spheroplast preparations. For the isolation of intact spheroplasts, EGTA-washed cells were treated essentially as described by Biggins [5]. A 2-h lysozyme digestion was carried out at 37°C in MES/NaCl buffer, containing 0.4 M of either sucrose or mannitol, 50—100 μg chlorophyll per ml, and 0.05% lysozyme (muramidase, Sigma). Microscopic examination indicated the absence of intact cylindrical filaments, and their replacement by round spheroplasts, occurring singly or in short chains. Further purification steps [5] were therefore omitted. About 80% of the spheroplasts were intact at the end of assay in stabilizing media, as determined by fluorometric measurement of released phycocyanin.

Spheroplasts were lysed by 30—300-fold dilution in the usual MES/NaCl buffer at 8 mg albumin per ml in the presence or absence of divalent cation. Microscopic examination revealed that lysis was virtually complete under these conditions.

Assay of oxygen evolution activity. An oxygen luminometer [6] of improved design was used throughout [7]. This instrument measures oxygen evolution rates as low as 100 nmol per mg chlorophyll per h, and oxygen flash-yields as low as 3 nmol per mg chlorophyll, each with a signal/noise ratio of 3 : 1. Determinations were made at a background oxygen level of 10 ppm, requiring a 15-min dark degassing period subsequent to the mixing of salts, Hill reagent, and preparation.

The standard assay mixture contained 10 mM MES/NaOH, pH 6.2, 30 mM NaCl, and in a total volume of 2.1 ml, 16 mg albumin, 10—20 μg chlorophyll from a thawed French-press aliquot, and 0.30 mM each potassium ferri- and ferrocyanide. Ferrocyanide was added to stabilize the redox potential, which affects oxygen evolution rate [2,3]. In critical applications, the ferrocyanide level was adjusted to maintain a uniform potential in samples differing in $CaCl_2$ concentration. Components of the assay mixture were combined just before the onset of degassing.

Illumination. Unless otherwise specified, degassed samples were illuminated with continuous light until the measured oxygen evolution rate attained a maximum, a process limited by the 90 s response time of the luminometer. Two Zeiss heat filters, various neutral density screens, and either a chromate or ferricyanide solution interceded between the sample and the 650 W tungsten lamp of a Braun projector. Proximity to light saturation, always greater than 95% in the data reported, was determined by stepwise increase in light intensity effected by replacement of the screens. Rates of oxygen evolution determined by this method were not corrected for losses caused by intense light. Extrapolation of loss rate to the onset of illumination suggested that initial activities were some 10—20% higher than those actually measured.

In some cases, samples were illuminated with a 10—20-s pulse of saturating light (heat filtered, from a 750 W Leitz-Prado projector). The resulting peak in the luminometer output was integrated and an average oxygen evolution rate calculated. In these experiments, a second pulse provided an estimate of the

rate of inactivation, and permitted calculation of initial activity. Reported data have been corrected for losses only when the correction exceeded 10% of the observed rate. Increasing the duration of the pulse failed to increase the calculated rate of oxygen evolution, establishing the absence of appreciable induction effects during the first minute of illumination.

Flash-yield experiments were carried out using trains of 120 flashes of saturating intensity, 10 μs duration, and interflash interval of 0.5 s. Light sources were a Strobrite lamp (U.S. Scientific Instruments, No. 3015) and three Stroboslave units (Gen Rad, No. 1593A) triggered simultaneously.

Chlorophyll determinations. The chlorophyll content of whole cells and spheroplasts was determined by methanol extraction as described by Mackinney [8]. The chlorophyll concentration of suspensions of French-press preparations was routinely determined by absorption at 680 nm using an extinction of 0.10 ml per $\mu g \cdot cm$. The latter value was obtained by centrifuging (130 000 $\times g$, 30 min) a representative suspension of known 680 nm absorbance and extracting the chlorophyll of the resulting pellet with methanol. The routine 680 nm determination had an uncertainty of ±5%, an error which affects only comparisons between different preparations. Unless otherwise specified, a single preparation was used in the experiments presented in any one table or figure.

Calcium determinations. The total calcium content of *P. luridum* was determined on a per-chlorophyll basis by atomic absorption analysis of French-press preparations in the isolation medium plus 10% lanthanum diluent (final concentrations, 7 mM $LaCl_3$, 0.1 mM $SrCl_3$), using an Instrumental Laboratories 151 atomic absorption spectrophotometer and the 422.7 nm absorption line. To determine the distribution of total cell calcium between soluble and insoluble fractions, the preparation was centrifuged as described and the chlorophyll-free supernatant analyzed separately from the pellet. Resuspension of the latter was facilitated by the addition 0.01 volume of 2% sodium dodecyl sulfate. Subsequent foaming during aspiration was prevented by the addition of a trace of silicone antifoaming agent.

Redox-potential measurement. Solution redox-potential determinations were made using a Radiometer combination platinum-calomel electrode and PHM-64 meter. Values are presented as mV vs. standard hydrogen electrode, 240 mV higher than the measured potentials [9].

Data presentation. The fraction of oxygen evolution rate or flash-yield which is dependent upon added Ca^{2+} will be designated E_{Ca}, where

$$E_{Ca} = \frac{\text{rate (or yield) with } Ca^{2+} - \text{rate (or yield) without } Ca^{2+}}{\text{rate (or yield) with } Ca^{2+}}$$

An analogous measure of Mg^{2+} effects was also employed.

Results and Discussion

Ca^{2+} release from the French-press preparation

The observed influence of Ca^{2+} concentration on the oxygen evolution activity of *P. luridum* French-press preparations can be given the following interpretation: Ca^{2+} is sequestered in vivo within some intracellular compart-

ment at a concentration of 10^{-2}—10^{-1} M, that observed [1] to be optimal for the functioning of the oxygen evolution apparatus. After breakage, dependence upon added Ca^{2+} results from equilibration of the Ca^{2+}-containing compartment with the much larger volume of the suspending medium.

To test the above hypothesis, the fate of total cell calcium was examined after subjecting *P. luridum* to the French-press procedure known [1] to effect Ca^{2+} dependence of oxygen evolution. After breakage, the chlorophyll-containing, oxygen-evolving fraction was separated centrifugally from the inactive supernatant and the calcium content of each fraction determined by atomic absorption spectrometry (see Materials and Methods). The results indicate that of the total recoverable calcium (200—400 nmol/mg chlorophyll, depending upon culture age) 90% was present in the supernatant. Given this substantial, but not complete, release of cellular calcium, the preceding hypothesis would predict both the stimulation of oxygen activity by Ca^{2+} addition and the persistence of a low level of oxygen evolution activity in the absence of added Ca^{2+}, as observed. Since the calcium concentration of the supernatant fraction was $2 \cdot 10^{-5}$ M, residual calcium in the pellet was either sequestered, or was bound with a dissociation constant of approx. $2 \cdot 10^{-4}$ M.

Reversibility of the Ca^{2+} effect

If added Ca^{2+} restores oxygen evolution by replacing lost endogenous Ca^{2+}, then removal of the cation from its site of action in vivo must be reversible. This property was successfully demonstrated, as shown in Table I. Preparations which were exposed to Ca^{2+} for a period sufficient to cause maximal return of oxygen evolution activity were separated from their suspending media by centrifugation. Resuspension in Ca^{2+}-containing but not in Ca^{2+}-free buffer resulted in the return of a large fraction of the initial activity. This supports the contention that added Ca^{2+} reverses the effects of Ca^{2+} loss during cell breakage.

Recombining the pellet and original supernatant in the presence of Ca^{2+} did not result in an enhancement of oxygen evolution activity above that obtained using Ca^{2+}-containing buffer. This failure to find activating 'Hill factors' in the soluble fraction of the homogenate contrasts with the results of other workers studying cell-free cyanobacterial preparations [10,11].

TABLE I

REVERSIBILITY OF Ca^{2+} EFFECTS ON LIGHT-SATURATED RATES OF OXYGEN EVOLUTION

Samples of a French-press preparation were added to the usual MES/NaCl/bovine serum albumin buffer containing 3.8 mM $CaCl_2$, and after 12 min in darkness at 5°C, centrifuged at 130 000 × *g* for 30 min. Supernatants, free of chlorophyll, were removed, and pellets (volume approx. 100 μl) resuspended in the standard assay mixture with or without $CaCl_2$, as indicated.

	$CaCl_2$ (mM)	O_2 evolution (μmol/mg chlorophyll per h)
Control (no centrifugation)	0	<0.1
	3.8	8.2
Resuspended pellet	0.2	0.6
	3.8	5.6
Recombined pellet and supernatant	3.8	5.2

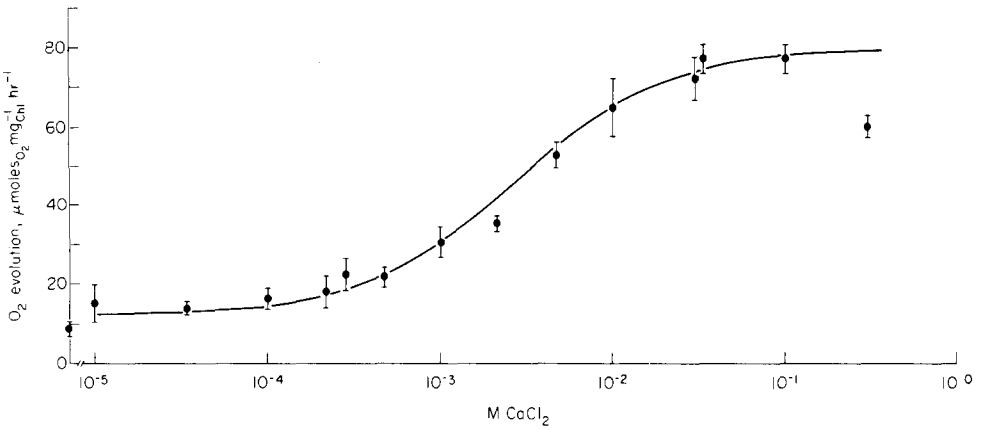

Fig. 1. Light-saturated rates of oxygen evolution as a function of concentration of added Ca^{2+}. The *P. luridum* French-press preparation was illuminated with 10 s of continuous light in the usual MES/NaCl/albumin buffer containing $CaCl_2$ as indicated, 13 μg chlorophyll per ml, 3.2 mM ferricyanide, and from 0.2 to 3.1 mM ferrocyanide, as necessary to establish a solution redox potential of 595 ± 10 mV vs. standard hydrogen electrode. Observed activity was corrected for loss (0—20%) incurred during flash preillumination (see Fig. 2). Error shown is due to uncertainty in this correction and in signal integration. Line is a calculated hyperbolic saturation curve, $ax/(b + x)$, scaled vertically by observed extrema, with a midpoint of 2.8 mM $CaCl_2$. The rate at 33 mM $CaCl_2$ was 21.4 nmol/mg chlorophyll per s.

Binding order and dissociation constant

A decline in oxygen evolution activity at high Ca^{2+} levels (ref. 1 and Fig. 1) suggests a model in which Ca^{2+} binding to one of two separate components facilitates the formation of an active complex; in excess Ca^{2+}, formation of the complex would be inhibited by the binding of Ca^{2+} to both components. Such a model predicts, however, that at fixed Ca^{2+} concentration, oxygen evolution per chlorophyll should increase with an increase in the assay concentration of the preparation. For the French-press preparation in 11 mM $CaCl_2$, the observed rates of light-saturated oxygen evolution at 0.25, 3.4, and 12.0 μg chlorophyll per ml were 22.5, 13.6 and 11.7 μmol/mg chlorophyll per h, respectively. This decline in activity with increasing chlorophyll concentration discounts any separate-subunit hypotheses, and also suggests the presence of some oxygen evolution inhibitors in the homogenate. The dependence of oxygen evolution upon [Ca^{2+}] was not appreciably affected by chlorophyll concentration (data not shown).

It is now possible to interpret an oxygen evolution rate vs. Ca^{2+} concentration curve as the reversible titration of a single Ca^{2+}-binding site. As shown in Fig. 1, fit of the observed rates to a simple 1 : 1 binding curve is quite good. Concentrations of added and free Ca^{2+} were taken to be identical, consistent with a low (15 μM) concentration of photosynthetic units (see below) and confirmed by measurements using a Ca^{2+} electrode (Radiometer). The maximal contribution to free Ca^{2+} by the added preparation is 10 μM, as determined by the calcium content of the preparation (see above).

The data of Fig. 1 were obtained from a preparation having particularly high maximal activity; however, the midpoint in the $CaCl_2$ titration, 2.8 mM, is quite close to that previously reported for a preparation of 10-fold lower maxi-

mal activity and more pronounced Ca^{2+} dependence [1]. It appears that the important features of the Ca^{2+} effect on oxygen evolution are unchanged by variations in the extent of irreversible activity loss or suppression of 'Ca^{2+}-independent' activity obtaining in particular preparations.

Unit activation and turnover facilitation

The rate of oxygen evolution under saturating continuous light is given by the product of two parameters; the number of active photosynthetic units, n, and the turnover rate constant, k. In order to discriminate between the effects of Ca^{2+} on each of these two terms, samples of the French-press preparation were illuminated with low frequency flashes of saturating intensity. Classically [12] the oxygen yield per flash under these conditions is identified with n, the number of photosynthetic units present and active. Using this assumption, we can then write,

$$\text{(continuous-light rate)/(flash yield)} = nk/n \tag{1}$$

$$nk/n = k \tag{2}$$

to obtain the turnover rate constant from continuous and single-turnover flash data.

Fig. 2 shows that Ca^{2+} increases the oxygen flash-yield. Evidently, units which were incapable of oxygen evolution after French-press treatment became active upon Ca^{2+} addition. The curve of Fig. 2 is similar to that of Fig. 1, the Ca^{2+} titration of oxygen evolution rate, but the E_{Ca} parameter calculated on the basis of yield (0.77) is less than that calculated from rate data of the same samples (0.88). Using Eqns. 1 and 2, it can be shown [7] that the difference in

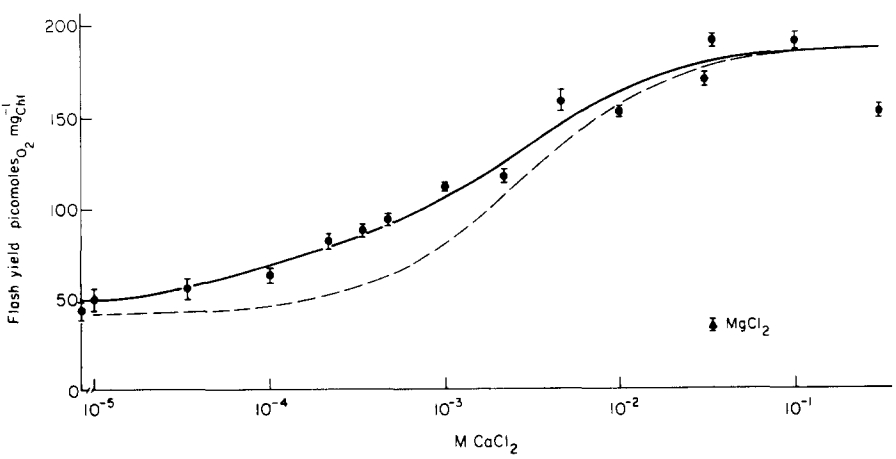

Fig. 2. Oxygen yield under saturating flash as a function of Ca^{2+} concentration. Samples were those of Fig. 1, illuminated with 120 flashes at 2 Hz. No correction was made for losses incurred during illumination, judged on the basis of a second, 120-flash sequence to be less than 8%. Error bars reflect uncertainty in signal integration only. Dotted line is the curve of Fig. 1. Solid line is given by a two-component equation, $ax/(b + x) + cx/(d + x)$, the two components having dissociation constants of 2.8 and 0.056 mM, and are present in a ratio of 3 : 1, respectively.

rate and yield E_{Ca} is due to a 2-fold more rapid turnover of Ca^{2+}-activated units vs. those evolving oxygen in the absence of added Ca^{2+}.

After scaling to the E_{Ca} observed under flash, the yield vs. Ca^{2+} concentration data of Fig. 2 fits only poorly the theoretical line of Fig. 1 (dotted line of Fig. 2). It is possible to fit the observed flash data with two Ca^{2+}-binding components (solid line of Fig. 2) the predominant species having a dissociation constant of 2.8 mM, the minor component having a 50-fold higher Ca^{2+} affinity. The absence of the high-affinity component from the oxygen rate vs. Ca^{2+} concentration curve (Fig. 1) is presumably due to its having a much lower rate of turnover than the major, 2.8 mM component; a low rate of turnover would weight unfavorably the contribution of the minor component to the total oxygen rate, but have no effect upon the oxygen yield under flash [7].

As previously reported [1], Mg^{2+} effects a 2—3-fold increase in oxygen evolution rate of *P. luridum* preparations under continuous light. Fig. 2 shows, however, that Mg^{2+} does not increase flash yield. From this it follows that Mg^{2+} facilitates turnover, but only of those units which are independent of added Ca^{2+}. This conclusion is supported by data [7] which indicate, in the presence or absence of Ca^{2+}, a constant increment in the oxygen evolution rate when Mg^{2+} is added (approx. 1 mol oxygen/mg chlorophyll per h at 10 mM $MgCl_2$). The Ca^{2+} requirement for the activation of photosynthetic units is therefore exceedingly specific.

Binding of Hill oxidant

It was important to determine whether the effect of Ca^{2+} on the rate of oxygen evolution was due to influence of the cation on the interaction of the preparation with the Hill oxidant [13]. Table II presents evidence against this non-physiological mechanism of Ca^{2+} action; across a broad range of Ca^{2+} concentrations, the rate of oxygen evolution is saturated with respect to the concentration of ferricyanide at 0.30 mM, the level of oxidant usually used. Insensitivity of oxygen evolution rate to a 10-fold increase in ferricyanide concentration indicates that the formation of a complex between ferricyanide and its site of reduction is limiting neither the number of active units nor their rate of turnover. Furthermore, because the ferricyanide and Ca^{2+} concentrations giving

TABLE II

EFFECTS OF Ca^{2+} ON OXYGEN EVOLUTION ACTIVITY AT VARYING CONCENTRATION OF HILL OXIDANT

P. luridum French-press preparations assayed in the usual MES/NaCl/bovine serum albumin buffer containing equal concentrations of ferri- and ferrocyanide. Solution redox potentials at high and low ferricyanide concentrations were within 15 mV of one another. Values are μmol O_2 evolved/mg chlorophyll per h.

$CaCl_2$ (mM)	Ferricyanide (mM)	
	0.3	3.0
0	2.2	2.2
3.8	4.6	4.6
29.0	11.0	10.5

TABLE III

Ca^{2+}-STIMULATION OF OXYGEN EVOLUTION IN THE PRESENCE OF UNCOUPLERS

Preparation No. 1 was assayed under continuous illumination in the steady state, with and without 7.2 mM $CaCl_2$. Preparation No. 2 was assayed, with and without 33 mM $CaCl_2$, under 10 s of continuous light, and correlated for losses as described in Materials and Methods. In all experiments, the standard assay buffer, and definition of E_{Ca} given in Materials and Methods, were employed.

	O_2 evolution (μmol/mg chlorophyll per h)		E_{Ca}
	No additions	With $CaCl_2$	
Preparation No. 1			
Control	1.0	11.3	0.91
Atebrin (10 μM)	1.0	13.2	0.92
CCCP (10 μM)	0.3	4.9	0.94
Preparation No. 2			
Control	6.7	26.0	0.74
Dinitrophenol	5.2	17.7	0.71

half-maximal oxygen evolution rates differ by more than an order of magnitude (<0.3 vs. 2.8 mM, respectively) formation of a Ca^{2+} · ferricyanide complex [14] cannot be limiting the oxygen evolution rate. If such a complex had been the only active Hill acceptor, the half-saturating concentrations of Ca^{2+} and of ferricyanide would have been identical. Thus, neither facilitation of ferricyanide binding, nor formation of a Ca^{2+} · ferricyanide complex can account for the observed stimulation by Ca^{2+} of oxygen evolution. This argues in favor of a role for Ca^{2+} within the native Photosystem II/oxygen evolution apparatus.

Effects of uncouplers

Table III demonstrates the persistence of Ca^{2+} stimulation in the presence of three substances known in other systems to uncouple electron transport from photophosphorylation [15]. Passive proton permeability, likely to obtain even in the control French-press preparation, is virtually assured by the addition of these proton carriers. The observed inhibition of oxygen evolution by CCCP and dinitrophenol would be expected at concentrations greater than those required for uncoupling [15]. Because the stimulation by Ca^{2+} is not diminished by uncouplers, the mechanism of stimulation cannot be neutralization by Ca^{2+} of transmembrane ion fluxes [16,17].

Variation of the preparation procedure

Table IV, line 1, indicates that the low rate of ferricyanide-coupled oxygen evolution from whole cells of *P. luridum* is not increased by the addition of Ca^{2+} to the suspending medium. Evidently, Ca^{2+} needed for oxygen evolution remains sequestered within the intact cell, even after washing with 1 mM EGTA.

A variety of procedures were employed with the intent of determining the degree of cell disruption required to bring about a dependence of oxygen evolution on added Ca^{2+}. Osmotically sensitive spheroplasts were useful in this respect [5]. Intact spheroplasts retain full photosynthetic capabilities [5,18] and have higher rates of ferricyanide-coupled Hill activity than whole cells,

TABLE IV

MAGNITUDE AND SPECIFICITY OF Ca^{2+} STIMULATION OF OXYGEN EVOLUTION IN VARIOUS PREPARATIONS OF *P. LURIDUM*

Whole cells were washed in MES/NaCl buffer containing 1 mM EGTA, and assayed in the same buffer without EGTA after addition of 4.3 mM each ferri- and ferrocyanide. Intact spheroplasts were prepared in MES/NaCl buffer containing 0.4 M mannitol, stored with minimal lysis at 0°C, and assayed after addition of 0.3 mM each ferri- and ferrocyanide, 20 μM A23187 (Eli Lilly and Co.) and 12 μM gramicidin D (Sigma). Ionophores were added in a small volume of dimethyl sulfoxide, which had no effect on control samples. French-press preparations, isolated in the presence of bovine serum albumin and stored in liquid nitrogen, were assayed in the standard assay mixture in the presence or absence of divalent cation. E_{Ca} and E_{Mg}, defined in Materials and Methods, were evaluated using activities at 11 mM $CaCl_2$ and 10 mM $MgCl_2$, respectively.

Sample	O_2 evolution * with $CaCl_2$	E_{Ca}	E_{Mg}
Whole cells	3.0	0.0	—
Intact spheroplasts + A23187, gramicidin	29—34	0.50—0.69	0.55
Lysed spheroplasts	24—40	0.75—0.90	0.12—0.45
French-press preparations	8—65	0.83—0.995	0.48—0.65

* μmol/mg chlorophyll per h.

presumably due to improved coupling to the exogenous oxidant. Table IV, line 2, indicates that intact spheroplasts, assayed in the presence of gramicidin D and ionophore A23187, show only a low, non-specific divalent-cation stimulation of oxygen evolution rate, i.e. $E_{Ca} = E_{Mg} = 0.5$. Under the assay conditions employed, the outer cell membrane should be highly permeable to divalent cations [19]. Lysis of spheroplasts results in the release of extrathylakoid material, including phycocyanin pigment, but preserves electron transport and photophosphorylation activities [18,20]. Although retention of photophosphorylation implies integrity of the thylakoid membrane subsequent to lysis, a transient increase of thylakoid permeability during lysis cannot be excluded. Table IV, line 3, indicates that lysed spheroplasts respond to the Ca^{2+} level of the suspending medium; $E_{Ca} = 0.75—0.90$. In the experiments of Table IV lysis was carried out at the divalent cation concentration obtaining during subsequent assay of oxygen evolution activity (see Conclusions).

Similar findings with lysed *P. luridum* spheroplasts have been reported by Binder et al. [18]. Like these workers, we have observed much smaller stimulatory effects of Mg^{2+} (Table IV, line 3). While these properties resemble those of French-press preparations, it is only in the latter that complete suppression of 'Ca^{2+}-independent' oxygen evolution is approached, and values of E_{Ca} in excess of 0.90 are obtained (Table IV, line 4). After French-press treatment, the ratio of total to Ca^{2+}-independent activities can reach 200 (i.e. $E_{Ca} = 0.995$).

High breakage pressure during the French-press procedure diminishes the total recoverable oxygen evolution activity; however, the degree of Ca^{2+} dependence is enhanced. Breakage at 100 vs. 24 000 lb/inch^2 yielded preparations with E_{Ca} values of 0.87 and 0.93, respectively. If *P. luridum* cells are pretreated with lysozyme so as to remove the outer cell sheath, an E_{Ca} value of 0.95 is obtained after breakage at only 1000 lb/inch^2.

If the French-press procedure is carried out in the presence of sucrose, activity determined in the absence of Ca^{2+} is preferentially stabilized, and E_{Ca}

TABLE V

SPECIES VARIATION OF Ca^{2+} AND Mg^{2+} STIMULATION OF LIGHT-SATURATED OXYGEN EVOLUTION

French-press preparations were assayed in the standard mixture, in the presence or absence of 11 mM divalent cation, and, where indicated, 100 μM EGTA, 4 μM DCMU. E_{Ca} and E_{Mg} were evaluated as in Table IV.

Species	O_2 evolution * with $CaCl_2$	E_{Ca}	E_{Mg}
P. luridum	10.6	0.96	0.65
An. flos-aquae	4.1	0.93	0.75
A. nidulans	26.0	0.84	0.61
C. vulgaris	26.0	0.52	0.57
C. vulgaris + EGTA	32.7	0.71	—
C. vulgaris + DCMU	0.5	0.9	—0.7

* μmol/mg chlorophyll per h.

falls [7]. The effects of sucrose may be related to other reports of stabilization of blue-green algal photosynthetic membrane by high levels of inert solutes [10,21,22].

Taxonomic variation

In Table V are shown the effects of Ca^{2+} and Mg^{2+} on the oxygen evolution activity preparations of two additional cyanobacteria, *Anabaena flos-aquae* and *Anacystic nidulans*. Together with *P. luridum*, the three species span most of the taxonomic spectrum of Cyanophyta [23]. In each preparation, the presence of Ca^{2+} is required for maximal oxygen evolution activity.

A French-press preparation of the green alga, *Chlorella vulgaris*, shows neither the magnitude nor specificity of the Ca^{2+} effects manifested in the cyanobacterial preparations. This result accords with other studies on eucaryotic preparations (see refs. 24—26, and references therein). Table V indicates, however, that the degree of Ca^{2+} dependence of *Chlorella* preparations can be increased by addition of EGTA or DCMU. The activity in the presence of the latter inhibitor is low but measurable by our sensitive method. This activity may arise from a severely disrupted fraction of the *Chlorella* preparation which has an exposed system II acceptor and low residual Ca^{2+} content.

Conclusions

The results presented here and in a previous report [1] strongly suggest that when the cyanobacterial photosynthetic apparatus is subjected to shear in dilute Ca^{2+}-free medium, sequestered Ca^{2+} is lost. This loss causes a dependence of the oxygen evolution activity on added Ca^{2+}. Reactivation by Ca^{2+} occurs via a reversible binding of the cation resulting in an increase in the number of oxygen-evolving Photosystem II reaction centers. The binding is predominantly characterized by a dissociation constant of approx. $3 \cdot 10^{-3}$ M, optimal activity obtaining in the range of $1 \cdot 10^{-2}$—$3 \cdot 10^{-2}$ M (Fig. 2).

The results of a comparative study of preparation procedures (Table IV) suggests that a breakdown of the external cell envelope is insufficient to effect a

complete release of Ca^{2+} important to oxygen evolution. Rather, French-press disruption of an intracellular compartment, presumably the thylakoid, is required for the full manifestation of Ca^{2+} dependence. The high pressure requirement may have its origin in protection by the cell sheath against complete rupture of the thylakoid.

If the thylakoid is the location of Ca^{2+} important to oxygen evolution, the concentration of free Ca^{2+} within this subcellular compartment should correspond roughly to the optimum in the activity vs. [Ca^{2+}] curve of Fig. 1. Assuming for *P. luridum* a ratio of chlorophyll to intrathylakoid volume equal to that of chloroplasts, viz., 3.3 μl per mg chlorophyll [27] and a total cell Ca^{2+} content of 300 nmol per mg chlorophyll (Table I), an intrathylakoid Ca^{2+} level of 30 mM requires that one-third of total cell Ca^{2+} be free within the thylakoid. While nothing is known of the compartmentation of cations within cyanobacteria, experiments with chloroplasts suggest very little of the total Ca^{2+} present in this organelle is free in the stroma [28,29]. This is consistent with an intrathylakoid localization of chloroplast Ca^{2+}. The failure of ionophore A23187 to bring about more than a modest, non-specific divalent cation dependence of oxygen evolution in intact *P. luridum* spheroplasts may indicate that the ionophore does not reach or act upon the enclosed thylakoids (Table IV).

The partial Ca^{2+} dependence of osmotically lysed spheroplasts (Table IV, line 3) may reflect a transient breakdown of the thylakoid membrane, resulting in partial equilibration of Ca^{2+} between intrathylakoid space and the external buffer. This would also explain the observation of Binder et al. [18] that Ca^{2+} added after spheroplast lysis was ineffective in increasing oxygen evolution activity. Presumably, the resealing of the thylakoid vesicles, which is required to account for photophosphorylation activity, prevents added Ca^{2+} from reaching its site of action.

The observations of other laboratories on the cation stimulation of cyanobacterial fragments can be largely accounted for by the data of Tables IV and V. Grinding procedures commonly employed [10,21,22] may well be too mild to bring about the release of Ca^{2+} we have observed using the French-press. One exception is a high-pressure shear preparation of *Nostoc* which shows rather high oxygen evolution activity in the absence of added Ca^{2+} [30] and, in the absence of monovalent salts, a 10-fold stimulation by Mg^{2+} [31]. The difference between this and other preparations may reflect physiological diversity within the cyanobacteria, at least with regard to the security of sequestered Ca^{2+}.

The maximal oxygen evolution rate reported here for the Ca^{2+}-dependent French-press preparation of *P. luridum*, coupled to ferricyanide, is 84 μmol oxygen/mg chlorophyll per h (Fig. 1). For whole cells coupled to CO_2 the rate is 300 μmol/mg per h [2]. These figures show that at optimal Ca^{2+}, 30% of the activity present in vivo can be recovered from the cell-free preparation. Higher activities can be obtained if Ca^{2+} is present during breakage [3,7] indicating that Ca^{2+} both stabilizes and activates oxygen evolution activity. Analogous stabilizing effects of Mg^{2+} on *Nostoc* preparations have been reported by McSwain et al. [31].

Recovery of photosynthetic activity can also be assessed on the basis of the number of photosynthetic units which we have taken to be the oxygen yield

under saturating flash illumination. The data of Fig. 2 give a maximal concentration of photosynthetic units of one per $5.6 \cdot 10^3$ chlorophylls. This ratio, when compared to a value of one per $1.7 \cdot 10^3$ in intact cells [32] indicates a 30% recovery of photosynthetic units. As this recovery is close to that calculated on the basis of oxygen evolution rate, use of Eqns. 1 and 2 would indicate that whole cells and the maximally reactivated preparation have approximately equal turnover times (8—10 ms).

Unit activation and turnover rate

Implicit in our use of Eqn. 1 is the identification of the observed flash yield with n, the effective number of units functioning under continuous illumination. While this identification has been almost universally assumed to be valid, one can envision circumstances in which it is not. For example, the high flux densities required for saturation by single flash may inhibit oxygen evolution [33], an effect which might not be obtained under lower flux densities sufficient for saturation by continuous light. Experimentally, this would be seen as a discrepancy between the value of the turnover constant calculated from an oxygen evolution rate/yield ratio (Eqns. 1 and 2) and that obtained directly from double-flash methods [32]. The French-press preparation described here is too unstable for precise double-flash work; however, preliminary experiments indicated a 3—4-fold slower turnover than had been estimated using the rate/yield calculation. This in turn means that the single-flash yield underestimated the number of photosynthetic units active under continuous illumination. Using the directly determined value for k, and the relation $nk/k = n$, where nk equals the rate of oxygen evolution under saturating, continuous light, we obtain a nearly 100% recovery of photosynthetic units at optimal Ca^{2+}. Further experiments are needed to support these preliminary findings.

In a following paper [3] the Hill acceptor selectivity and redox potential sensitivity of the *P. luridum* French-press preparation will be examined in detail. These data, in addition to the DCMU insensitivity previously reported [1], strongly suggest the operation of an abbreviated electron transport pathway, involving only events on the oxidizing side of the DCMU block. That Ca^{2+} is required for the function of this pathway, but is not specifically involved with the terminal steps of ferricyanide binding, supports the claim that the role of Ca^{2+} in vivo is within the Photosystem II reaction center or the oxygen evolution apparatus.

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