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Photosynthetic Electron Transfer in Preparations of the Cyanobacterium *Spirulina platensis*[1]

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ABSTRACT

Electron transfer activity in intact trichomes of *Spirulina platensis* (Nordst.) Geitl. can be observed with either CO_2 or methylviologen as the Hill acceptor. Ferricyanide cannot penetrate the intact trichomes, but photoreduction of this oxidant can be observed when mediated by lipophilic oxidants such as *p*-phenylenediamine or 2,5-dimethyl-*p*-benzoquinone. The insensitivity of these reactions to dibromothymoquinone indicates that they are due largely to the activity of photosystem II. Direct photoreduction of ferricyanide can be observed in spheroplasts of *Spirulina*, indicating that such preparations have altered permeability properties when compared with intact trichomes. Preparation of these spheroplasts, which are osmotically fragile, requires that intact trichomes be washed with KCl and EDTA to induce lysozyme sensitivity and thereby allow digestion of the cell wall. The KCl/EDTA washing procedure used for spheroplast preparation alters the permeability of *Spirulina* trichomes, as evidenced by the ability of these preparations to photoreduce ferricyanide. This photoreduction reaction is insensitive to dibromothymoquinone, and is stimulated by high concentrations of divalent cations. During assays, the reaction is inhibited by the inclusion of polyethyleneglycol as an osmotic protectant. Photoreduction of methylviologen and $NADP^+$ is also observed in the washed trichomes, along with an endogenously catalyzed photoreduction of O_2 to H_2O_2. Photophosphorylation cannot be observed in the washed preparations, but cyclic photophosphorylation with phenazinemethosulfate is observed after mild sonication. These results indicate that KCl/EDTA-washed trichomes of *S. platensis* retain the full range of energy transducing capacities associated with thylakoid membranes of the intact trichomes; the washing procedure facilitates spheroplast formation and alters, but does not abolish, permeability barriers in these preparations.

Electron transfer activity in cyanobacteria can be studied in intact cells using either CO_2 or artificial mediators as electron acceptors (5, 35). Exposure of intact cells to lysozyme digestion will, in some cases, produce spheroplasts to provide an alternative method for studying electron transfer activity without resorting to drastic methods of cell disruption (5–8, 30). Mechanical disruption can also be used to produce membranous vesicles derived from cyanobacterial thylakoid membranes (11, 21, 32, 36). Each of these methods has certain merits. Intact cells provide a physiologically unaltered system, but are also the most difficult to probe experimentally. With spheroplasts, a somewhat simpler system is produced in which the cell wall is no longer present as a potential barrier to solute permeation, and carefully isolated spheroplasts retain the ability to evolve O_2 with CO_2 as the Hill acceptor. However, spheroplast production has been obtained with very few cyanobacteria, and even in these preparations permeability barriers still exist so that some reactions, such as photophosphorylation, can not be directly monitored. Vesicles, although unable to conduct CO_2-dependent reactions, are a much simpler system in which the cyanobacterial electron transfer system is accessible to assay using the widest range of Hill oxidants, including ferricyanide, MV[3] and Fd/$NADP^+$ (21, 36). With vesicles, however, one must question whether physical shock has created an artifactual perturbation of membrane architecture.

We have been engaged in studies characterizing photosynthetic electron transfer under various conditions of cell disruption of *Spirulina platensis*. This cyanobacterium is of considerable interest because of its importance as a source of edible protein (23) and because of investigations on the properties and sequences of certain of its electron transfer components (2, 14). Furthermore, this organism was used for isolation and purification of Ca^{2+}-ATPase, and the first unambiguous demonstration of a cyanobacterial coupling factor (22). We report here that washing of trichomes with hypertonic KCl plus EDTA facilitates spheroplast formation and alters the permeability of *S. platensis* to artificial mediators of electron transfer. Photoreduction of ferricyanide is shown to be supported by PSII, and is subject to regulation by added cations. In addition, we present evidence for photosynthetic reduction of other exogenously added mediators, including $NADP^+$, as well as results of experiments to assay photophosphorylation in these KCl/EDTA-permeabilized preparations.

MATERIALS AND METHODS

Culture Conditions. Axenic cultures of *Spirulina platensis* (Nordst.) Geitl., clone OPL, were supplied by Dr. Susan Kilham. Cells were grown in the medium of Zarrouk (39), supplemented with 2.5 ml/l of the micronutrients of Allen (1). Growth was in batch cultures in 4-liter Erlenmeyer flasks at 35°C with agitation provided by bubbling with filtered, humidified air; illumination was provided by a bank of six to eight Sylvania 'Lifeline' fluorescent lamps located approximately 0.305 m above the tops of the culture flasks.

Intact Trichomes. Late log-phase cultures were harvested by centrifugation of 200- to 400-ml batches at 10,000*g* for 20 min and suspended in 80 ml of a solution containing 7.5% PEG-4000, 1 mM $CaCl_2$, and 20 mM Hepes-NaOH (pH 7.5; 'resuspension

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[3] Abbreviations: MV, methylviologen; DAD, diaminodurene; DMBIB, dibromothymoquinone or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DMQ, 2,5-dimethyl-benzoquinone; PD, phenylenediamine; PMS, phenazinemethosulfate.

medium') using a Teflon cell homogenizer. The suspended trichomes were then treated for 1 min with a Waring Blendor fitted with a Cenco semimicro stainless steel chamber; this treatment broke the long trichomes into shorter filaments, permitting easier manipulation during subsequent assays without affecting the activity or permeability of the trichomes (data not shown). The blended filaments were pelleted by centrifugation for 5 min at 3,000*g* and resuspended in a minimal amount of the resuspension medium. After the Chl concentration was determined, the intact trichomes were used for assay.

EDTA/KCl-Washed Cells. Pellets of harvested trichomes were suspended in approximately 80 ml of 10 mM EDTA, 10 mM Hepes-NaOH (pH 7.5); in some of the preparations, 5.0% or 7.5% PEG was included at this stage; this addition had no effect upon subsequent activities (data not shown). The trichomes were centrifuged for 5 min (3,000*g*), the loose material atop the pellet was discarded, and the pellet was rinsed and resuspended. This suspension was then given two 1-min bursts with the Waring Blendor, with care taken to ensure that the temperature did not rise above 25°C. The shortened filaments were pelleted by centrifugation (3,000*g*), resuspended in a solution of 1.5 M KCl, 5 mM EDTA, 10 mM Hepes-NaOH (pH 7.5), stirred for 5 min at 4°C, and centrifuged (3,000*g*). The pellet was then resuspended in a minimal amount of the initial resuspension medium, the Chl concentration was determined, and the preparation was used for assays.

Spheroplasts. Cells washed with EDTA/KCl were prepared as described above, pelleted by centrifugation, and resuspended in 50 ml of a medium containing 10% PEG, 10 mM Tricine-NaOH (pH 8.0), 2 mM $MgCl_2$, 2 mM $CaCl_2$, 40 mM NaCl, and 0.01% (w/v) lysozyme. This suspension was shaken gently at 35°C in a water bath. Small aliquots were taken frequently to be observed microscopically and tested for osmotic fragility. Spheroplast formation corresponds to a breakage of the filaments into single cells and the appearance of osmotic fragility, which could be detected by resuspension of the spheroplasts in a hypotonic medium devoid of PEG; if the lysozyme digestion had been effective, the cells ruptured and the supernatant after centrifugation contained the characteristic blue color of phycocyanin. When greater than 90% of the cells appeared by these criteria to be spheroplasts (45–90 min), the preparation was collected by low-speed centrifugation, washed once, and resuspended, using a Vortex mixer, in a minimal amount of medium consisting of 7.5% PEG, 10 mM $CaCl_2$, 5 mM $MgCl_2$, and 20 mM Hepes-NaOH (pH 7.5). This preparation was maintained on ice until assay.

Chl Determination. For Chl determination, cells were diluted in H_2O and sonicated in an ice bath for 1 min using the microtip of a Branson W185 sonicator at a power setting of 4. Acetone was then added to a final concentration of 80% (v/v), and the Chl concentration was determined by *A* at 663 nm (20).

Electron Microscopy. Harvested pellets of intact or EDTA/KCl-washed trichomes of *S. Platensis* were fixed using 2% glutaraldehyde followed by 1% OsO_4. Staining in 2% uranyl acetate was performed *en bloc*. The fixed preparations were then embedded in Spurr's low-viscosity epoxy resin, sectioned, and stained with lead citrate. The sections were examined and photographed using a Zeiss 9S-2 electron microscope.

Assays of Electron Transfer. Assays of electron transfer activities were conducted using a 1.5 ml thermostatted (25°C) glass cuvette, fitted with a Clark-type O_2 electrode (Yellow Springs Instrument Co.) with battery-powered polarizing and bridge circuits (10). Illumination was provided by an Oriel model 6325 light source; the light was passed through a heat filter comprised of a 0.2% $CuSO_4$ solution (5-cm light path) and a red filter (cut-on at 600 nm); light intensity at the surface of the reaction vessel was approximately 10^6 ergs cm^{-2} s^{-1}.

The reaction mixture for assays of electron transfer consisted of 25 mM Hepes-NaOH (pH 7.5), 20 mM NaCl, and 20 to 30 μg Chl per assay. Other additions are noted in the legends for figures. It was important that cell preparations be thoroughly agitated (using a Vortex mixer) before an aliquot was taken for assay; otherwise, settling caused large sampling errors in the amount of biological material present. Certain other reactions were monitored by meas-

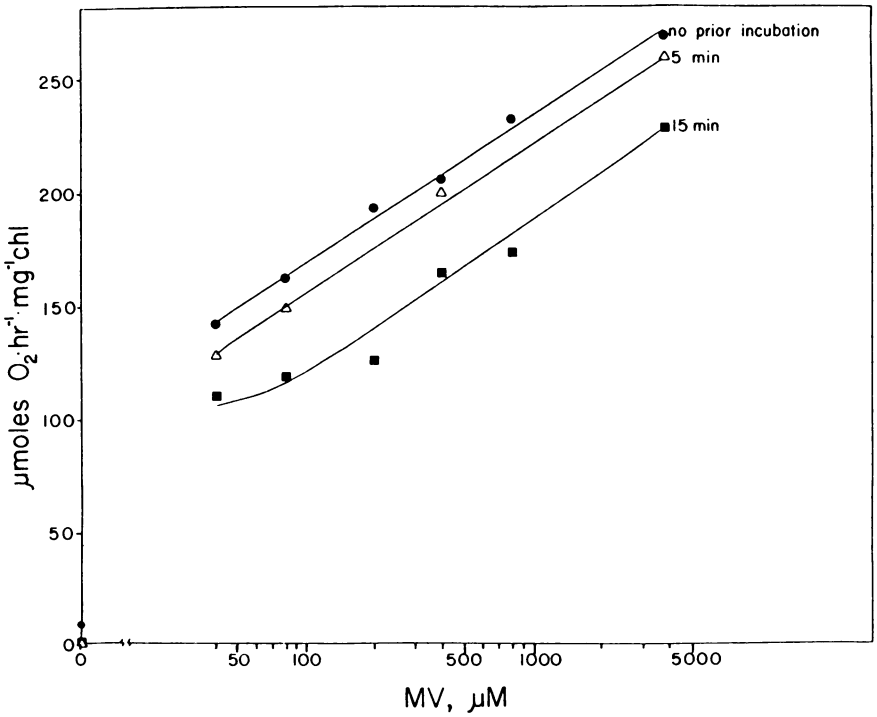

FIG. 1. The effect of MV concentration and time of incubation upon photosynthetic O_2 uptake in intact trichomes. Intact trichomes were incubated in the dark in various concentrations of MV, then assayed after the indicated intervals, using the assay conditions detailed in "Materials and Methods." During assay, 1.25 mM KCN was present.

uring reaction products at the end of a period of illumination. Production of H_2O_2 was measured by adding 40 μl of catalase (2 mg/ml) to the stoppered cuvette; O_2 evolution resulting from dismutation of H_2O_2 was monitored directly by the O_2 electrode. Production of NADPH was measured by removing the contents of the cuvette after illumination and quickly pelleting the cells by centrifugation. The supernatant *A* was measured 340 nm, PMS (6.5 μM) was added, and the change in *A* at 340 nm was used to determine the NADPH concentration (33).

Photophosphorylation Assays. The reaction mixture for determination of photophosphorylation contained 20 mM Hepes-NaOH (pH 8.0), 20 mM NaCl, 10 mM $MgCl_2$, 5 mM ADP, 5 mM NaH_2PO_4 (containing 10^6 cpm ^{32}P), 100 μM PMS, and 3 μM DCMU, along with a sample of *S. platensis* equivalent to 15 to 20 μg Chl. The cyclic photophosphorylation reaction was conducted in test tubes immersed in a water bath at 25°C. Illumination by white light (10^6 ergs cm^{-2} s^{-1}) was provided by photoflood lamps. Following illumination, the reaction mixtures were quenched by addition of TCA, and the unreacted phosphate was removed by complexation with ammonium molybdate and extracted with isobutanol-toluene; the aqueous phase was dried on planchettes for gas flow counting. ATP synthesis was determined using the method of Avron (4).

Materials. Hepes buffer was obtained from Sigma Chemical Co., as were catalase and lysozyme; MV was from Aldrich. Eastman supplied DMQ and PD, and DAD was from Research Inorganics; these three electron mediators were recrystallized according to the method of Gould (13). DCMU was obtained from K + K Labs, and was recrystallized by aqueous precipitation from ethanolic solution. DBMIB was a gift from J. N. Siedow. All other chemicals were of the highest grade commercially available.

RESULTS

Properties of Electron Transfer of Intact *Spirulina* Trichomes. Like other cyanobacteria, intact trichomes of *S. platensis* are capable of CO_2-dependent O_2 evolution; the rates range from 150 to 175 μmol O_2 h^{-1} mg^{-1} Chl (data not shown). *Spirulina* trichomes are also capable of utilizing cationic MV as a Hill acceptor without the dark incubation period necessary in some other cyanobacteria (9) to allow for penetration of the viologen dye into the trichome. As shown by the data of Figure 1, low concentrations of MV support electron transfer at rates equal to those obtained with CO_2. Preincubation with the dye lowers these rates; increased MV concentrations produce higher rates of O_2 evolution, but in no case does a dark preincubation period produce activity which equals or exceeds the rate of O_2 evolution obtained when the preincubation step is omitted. Both CO_2- and MV-supported activities are sensitive to DCMU and DBMIB, which are inhibitors of chloroplast electron transfer.

Although trichomes of *S. platensis* seem to be freely permeable to a cationic acceptor (MV) under our conditions of assay, attempts to obtain photoreduction of the anionic oxidant ferricyanide were unsuccessful. This finding was not surprising in view of results which have shown ferricyanide to be impermeant to intact, class I chloroplasts (34). We therefore sought to couple photosynthetic electron transfer activity in intact trichomes to ferricyanide reduction using lipophilic oxidants (17, 27). These results are presented in Figure 2, where increasing concentrations of PD and DMQ can be shown to elicit O_2 evolution activity in intact trichomes in the presence of ferricyanide. Note that the rate-saturating concentrations of these acceptors are not appreciably greater than those required for optimal activity with isolated eukaryotic thylakoids, with PD acting as a more effective acceptor than DMQ. Experiments to assess the ability of DBMIB to inhibit photoreduction of DMQ and PD in trichomes showed that these reactions were insensitive to low concentrations of DBMIB below 0.5 μM, although substantially higher DBMIB concentrations (>2.0 μM) produced inhibition (data not shown). These observations, which are very similar to those reported for isolated chloroplast thylakoid membranes (18), show that PD and DMQ are reduced predominantly by PSII in *Spirulina* trichomes, and that DBMIB can also act as a class III acceptor, supplanting the acceptor activities of PD or DMQ.

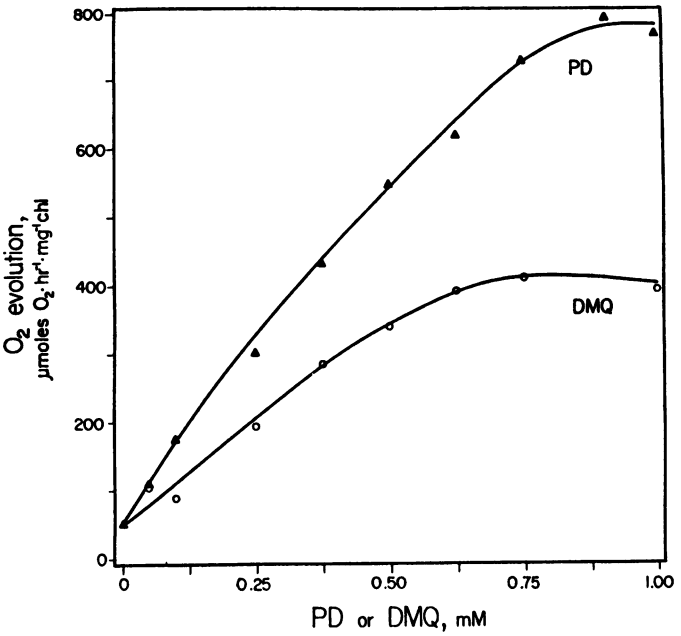

FIG. 2. The effects of the class III acceptors, PD and DMQ, upon photosynthetic O_2 evolution in intact trichomes. Assays were performed as described in "Materials and Methods," using 2.5 mM $K_3Fe(CN)_6$ and the indicated concentrations of *p*-phenylenediamine (PD) or 2,5-dimethylquinone (DMQ).

Table I. *Summary of Electron Transfer Activities in Intact Trichomes*

Various mediators or substrates for electron transfer are listed, showing the optimal concentrations for assay, the photosystem(s) assayed in each case, and typical rates of electron transfer.

Mediator	Concentration	Reaction	Rate[a]
			μmol O_2 h^{-1} mg^{-1} Chl
$NaHCO_3$	125 mM	PSII + PSI	150–175
MV	125 μM	PSII + PSI	150–175
$K_3Fe(CN)_6$	2.5 mM		0[b]
DAD	0.75 mM	PSII[c]	390–450
DMQ	0.75 mM	PSII[c]	380–420
PD_{ox}	0.75 mM	PSII[c]	450–770
Ascorbate/DAD → MV	6.0 mM asc, 0.5 mM DAD	PSI[d]	500–600

[a] Rates with MV are O_2 consumption; all others are O_2 evolution.
[b] No stimulation over the low endogenous rate.
[c] DBMIB insensitive, DCMU sensitive.
[d] DCMU insensitive.

A summary of the electron transfer activities of intact trichomes are presented in Table I. The striking feature of these data is the similarity to results which are obtained in assays of intact class I chloroplasts (34). O_2 evolution is supported by Calvin cycle activity with CO_2 as the substrate, and ferricyanide-supported Hill activity cannot be obtained under any nondisruptive conditions. Addition of MV to the trichomes abolishes CO_2 reduction and establishes a Mehler reaction, whereas electron transfer from the reducing side of PSII is linked to ferricyanide by the mediation of lipophilic oxidants. In addition, the PSI-dependent electron trans-

Table II. *Effects of EDTA Washing, Followed by Hypertonic Shock in KCl, upon Photosynthetic Activities of Trichomes*

Cells were washed in EDTA, then exposed to the various KCl concentrations indicated or as described for the EDTA/KCl-wash procedure in "Materials and Methods." Assay mixtures contained either 125 mM $NaHCO_3$, or 2.5 mM $K_3Fe(CH)_6$ (plus 0.75 mM PD). Where indicated, 100 mM $MgCl_2$ and 100 mM $CaCl_2$ were added to the assay mixture.

Assay	Treatment			
	EDTA, no KCl	EDTA, 0.5 M KCl	EDTA, 1.0 M KCl	EDTA, 1.5 M KCl
	μmol O_2 h^{-1} mg^{-1} Chl			
$H_2O \rightarrow CO_2$	162	93	29	−40[a]
$H_2O \rightarrow Fe(CN)_6$	0	0	0	−31[a]
$H_2O \rightarrow Fe(CN)_6 + Mg^{2+}, + Ca^{2+}$	12	31	83	145
$H_2O \rightarrow PD_{ox}$	353	310	127	120
$H_2O \rightarrow PO_{ox} + Mg^{2+}, + Ca^{2+}$	537	262	178	181

[a] Rates of O_2 consumption, rather than evolution.

Table III. *Comparison of the Electron Transfer Activities of Spirulina Preparations*

Assays conducted as explained in "Materials and Methods," with 100 mM $MgCl_2$ or 10% PEG present during the indicated assays. Values are photosynthetic rates of net O_2 change.

Assay Conditions	Untreated Trichomes	KCl/EDTA-Washed Trichomes	Spheroplasts
	μmol O_2 h^{-1} mg^{-1} Chl		
$H_2O \rightarrow CO_2$	160	−53[a]	0
$H_2O \rightarrow K_3Fe(CN)_6$	−20	−100	0
$H_2O \rightarrow K_3Fe(CN)_6$ + PEG	0	−125	80
$H_2O \rightarrow K_3Fe(CN)_6 + Mg^{2+}$	15	100	−17
$H_2O \rightarrow K_3Fe(CN)_6$ + PEG, $+ Mg^{2+}$	17	10	140
$H_2O \rightarrow PD_{ox}$	280	507	0
$H_2O \rightarrow PD_{ox}$ + PEG	267	371	112
$H_2O \rightarrow PD_{ox} + Mg^{2+}$	335	388	−12
$H_2O \rightarrow PD_{ox}$ + PEG, $+ Mg^{2+}$	246	269	103

[a] Negative values indicate O_2 consumption, rather than evolution.

fer reaction ascorbate/DAD → MV can be observed in these preparations. Thus, without resorting to cell disruption, the range of site-specific electron transfer activities characteristic of oxygenic photosynthetic systems can be conveniently assayed in *S. platensis*.

Preparation and Assay of *S. platensis* Spheroplasts. The data presented above clearly demonstrate barriers to permeation of ferricyanide in intact *S. platensis* trichomes, a barrier function imposed perhaps by the gram-negative cell wall and outer membrane structures of this cyanobacterium. These structures have been removed from other cyanobacteria by lysozyme digestion to produce permeaplasts (36) or osmotically fragile spheroplasts (5, 30). We have employed a number of treatments with *S. platensis* trichomes in an effort to obtain spheroplasts, and have determined that, without prior treatment of trichomes, lysozyme digestion cannot be obtained. Several procedures used with other bacteria (exposure to high pH, dilute toluene [3], or Tris [38]) induce lysozyme sensitivity of *S. platensis*; however, with none of these treatments can photosynthetic O_2 evolution be maintained (data not shown). We finally devised a simple procedure for induction of lysozyme sensitivity and retention of O_2 evolution using the EDTA/KCl wash described in "Materials and Methods." The effects of this procedure on *S. platensis* are complex, as indicated by the data presented in Table II, where the washing procedure produces an enhanced permeability of the trichomes to ferricyanide, as evidenced by the appearance of O_2 evolution in the presence of this oxidant. In addition, with increasing concentrations of KCl, CO_2-dependent Hill activity is lost and replaced by a slow O_2 uptake reaction. Concomitantly, O_2 evolution monitored by the PD-mediated reaction is seen to decrease, although some of this activity can be restored by addition of divalent cations (Mg^{2+}, Ca^{2+}) which may have been depleted by the EDTA wash procedure.

When trichomes are exposed to this KCl/EDTA wash procedure and subsequently incubated with lysozyme, osmotically fragile spheroplasts are produced. By varying the content both of PEG and of divalent cations in the suspending medium, it is possible to retain phycocyanin and to assay O_2 evolution activity with ferricyanide as the Hill acceptor. Among a variety of osmotic protectants we have examined during these experiments (sucrose, mannitol, sorbitol, Ficoll, spermine, BSA), only PEG has been found capable of maintaining the integrity of the spheroplasts (data not shown). Table III summarizes the results of electron transfer assays with the spheroplasts; data for intact and for KCl/EDTA-washed trichomes are included for purposes of comparison. As expected from the results presented above (Table II), the spheroplasts do not retain any CO_2-dependent O_2 evolution activity. Additionally, ferricyanide-dependent O_2 evolution is dependent on maintainance of spheroplast integrity by PEG. This O_2 evolution is not dependent on the presence of PD, nor does it show an absolute requirement for a divalent cation.

Further Characterization of Electron Transport in Permeabilized *Spirulina* Trichomes. The data of Tables II and III show that exposure of intact *Spirulina* trichomes to washing with KCl and EDTA induces a cation-dependent ferricyanide reduction reaction. To further explore this reaction, we assessed the extent to which the wash procedure altered the ultrastructure of *S. platensis*,

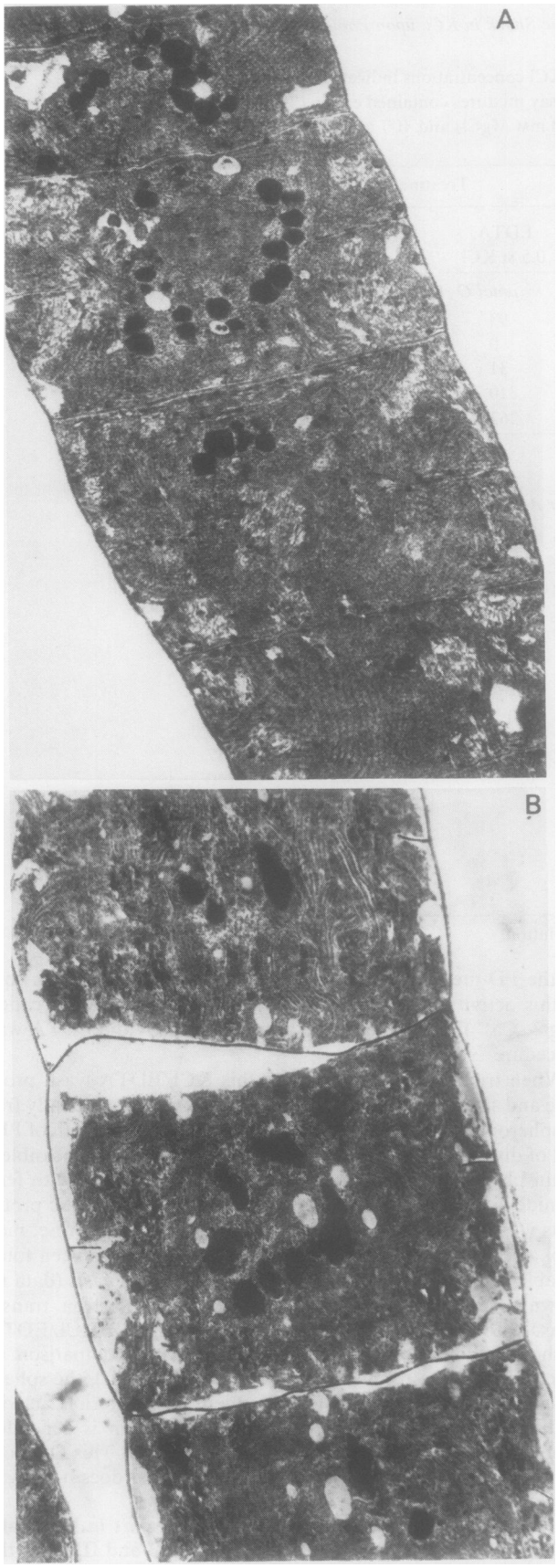

FIG. 3. Electronmicrographs of longitudinal sections through intact (A) and EDTA/KCl-washed (B) trichomes of *S. platensis*. A, × 13,900; B, × 17,300.

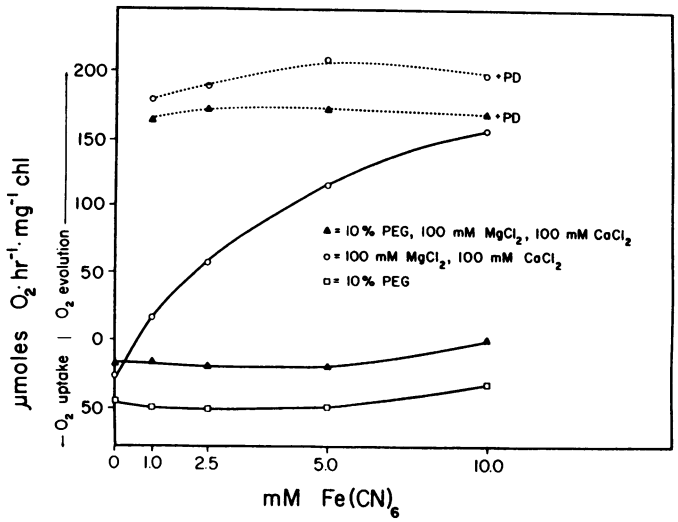

FIG. 4. Ferricyanide-supported electron transfer activity assayed in the presence of PEG and divalent cations. Assays were conducted on EDTA/KCl-washed cells in mixtures containing varied concentrations of $K_3Fe(CN)_6$ and the indicated additions to the standard reaction mixture (Table I). When added, PD was at a concentration of 0.75 mM.

using electron microscopy. Figure 3A shows a representative section of an intact trichome, where several outer layers, including the cell wall, enclose an internal thylakoid space (12, 15). Figure 3B shows the changes in ultrastructure produced by the EDTA/KCl washing procedure. Among these changes is evidence of plasmolysis along with changes in the appearance of the region of the cell wall. Note, however, that the structural integrity of the thylakoid membranes has not been altered by the wash procedure. These observations, along with the data of Tables II and III, suggest that although the photosynthetic apparatus of the washed trichomes is largely intact, the permeability properties of these preparations have been altered.

In Figure 4, data are presented which indicate that both an osmotic protectant (PEG) and ionic factors (the presence of a divalent cation) affect electron transfer in KCl/EDTA-washed trichomes when ferricyanide is the Hill oxidant. When PEG is present during assay of the trichomes, either with or without high concentrations of divalent cations (Mg^{2+} and Ca^{2+}), no O_2 evolution is observed. In the absence of PEG (with divalent cations present), a high rate of O_2 evolution can be obtained at high concentrations of ferricyanide. In other words, there is strong stimulation of ferricyanide-supported O_2 evolution if the proper ionic and osmotic conditions are provided; specifically, this activity is stimulated by divalent cations and retarded by PEG. Electron transfer to the lipophilic oxidant PD is, by comparison, unaffected by these same ionic and osmotic conditions (Fig. 4). These data indicate that the effect of PEG is to exclude ferricyanide from photoreduction sites in the EDTA/KCl-permeabilized trichomes. This view is reinforced by the observation that incubation of washed trichomes with ferricyanide prior to illumination is able to induce increased rates of O_2 evolution (data not shown). Therefore, we conclude that washed trichomes retain an osmotically sensitive barrier to the penetration of ferricyanide.

Although the data presented thus far show that EDTA/KCl-permeabilized trichomes can photoreduce added ferricyanide, they do not identify the site of photoreduction with either photosystem in these preparations. We therefore sought to resolve this question by assessing the sensitivity of ferricyanide photoreduction to inhibition. Figure 5 establishes that ferricyanide photoreduction is much less sensitive to DBMIB inhibition than is reduction of MV. Similar results with sonicated preparations of *Anabaena* and *Spirulina* have been reported (28, 36), and in agreement with these

investigators, we interpret our results to indicate that ferricyanide photoreduction in cyanobacterial membranes occurs predominantly at a site on the reducing side of PSII, prior to the DBMIB block.

The observations that PEG and divalent cations regulate access of ferricyanide to photoreduction sites near PSII, rather than PSI, might suggest that the thylakoids have been damaged by the EDTA/KCl wash procedure necessary to permeabilize *Spirulina* trichomes. To assess the possible extent of any such damage, we next examined an electron transfer reaction requiring labile membrane-associated components, namely, the photoreduction of $NADP^+$, which requires the presence of two protein components (Fd and Fd : NADP oxidoreductase) which are easily washed off of cyanobacterial and eukaryotic thylakoids (29, 31). The results of such experiments are depicted in Figure 6 (left) where increasing concentrations of $NADP^+$ are shown to be capable of decreasing the rate of O_2 uptake in EDTA/KCl-washed trichomes, indicating that added $NADP^+$ can penetrate these preparations to compete with the endogenous O_2 uptake reaction. This effect is countered by addition of MV, an autooxidizable acceptor. The O_2 uptake reaction, either in the presence or absence of exogenous acceptors, produces H_2O_2, an end product which can be assayed by dismutation in the presence of added catalase (Fig. 6). Thus, from determination of end product concentrations by spectrophotometry (for NADPH formation) or dismutation of peroxide (for endogenous autooxidation), and by polarographic assay of O_2 changes during photoreductions, total noncyclic electron flow can be quantitated. Using these methods, we sought to further characterize the effects of Mg^{2+}, to obtain an overview of the effects of a divalent cation on electron transfer to both artificial and native acceptors (MV, ferricyanide, $NADP^+$, O_2). These data are presented in summary in Table IV. A common effect of Mg^{2+} in all assays is the suppression of total noncyclic activity. At these concentrations of added cations, there is an apparent stimulation of ferricyanide activity measured as net O_2 evolution; this appears to be due to a decrease in the rate of the endogenous photoreduction of O_2 and increased preference for the pathway of electron flow to ferricyanide.

The finding that the EDTA/KCl-washed trichomes of *S. platensis* can support the photoreduction of exogenously added $NADP^+$ suggested to us that it might also be possible to observe photophosphorylation in these preparations, if ADP and phosphate were supplied. When this assay was attempted using MV as the acceptor in noncyclic assays, only low rates (7–25 μmol ATP h^{-1} mg^{-1} Chl) were achieved; the same was true of cyclic assays using PMS. Such results suggested that the trichomes might have retained a permeability barrier to the substrates for phosphorylation; if this were so, then minimal sonic disruption of the washed trichomes might remove this barrier and permit photophosphorylation assays. As shown by the data in Table V, this result was in fact obtained. Brief sonication of the washed trichomes produces a preparation capable of PMS-mediated photophosphorylation at rates of nearly 300 μmol ATP h^{-1} mg^{-1} Chl.

DISCUSSION

The data reported here characterize both the electron transfer and permeability properties of *Spirulina* preparations. Intact trichomes of this organism are permeable to MV (Fig. 1) DMQ, and PD (Fig. 2), but not ferricyanide. In this regard, the permeability properties of intact trichomes are quite similar to those of intact class I chloroplasts (34). It is somewhat surprising that MV should be accessible to PSI photoreduction sites in *Spirulina*; in studies with other cyanobacteria, Chua (9) demonstrated the necessity for prolonged incubation periods with high concentrations of MV to overcome a permeability barrier and permit maximal Mehler reaction activity. The absence of this barrier has permitted us to assay Mehler activity with either PSII and PSI, or with PSI alone using a lipophilic donor such as DAD (Table I).

In contrast to other cyanobacteria, such as *Phormidium luridum* (5, 6), *Spirulina* trichomes are unusually resistant to lysozyme digestion. The KCl/EDTA wash procedure we describe here not only permits cell wall digestion but also alters the permeability in the trichomes.

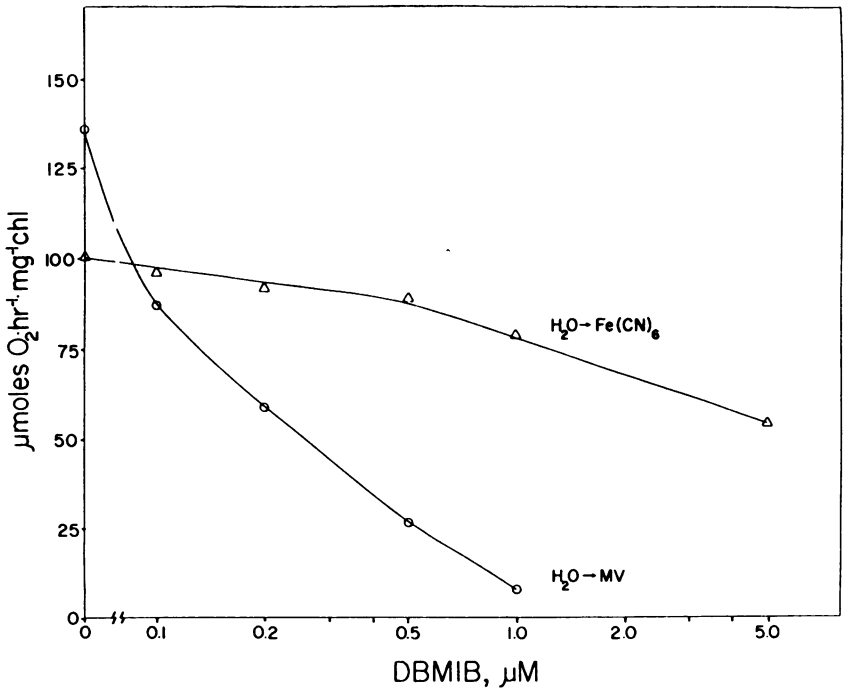

FIG. 5. The effects of DBMIB upon ferricyanide- and MV-supported photosynthetic activities in EDTA/KCl-washed trichomes. Assay mixtures contained 133 μM MV or 2.5 mM $K_3Fe(CN)_6$, and the indicated concentrations of DBMIB. The MV-dependent activity was measured as O_2 consumption; the ferricyanide activity as O_2 evolution.

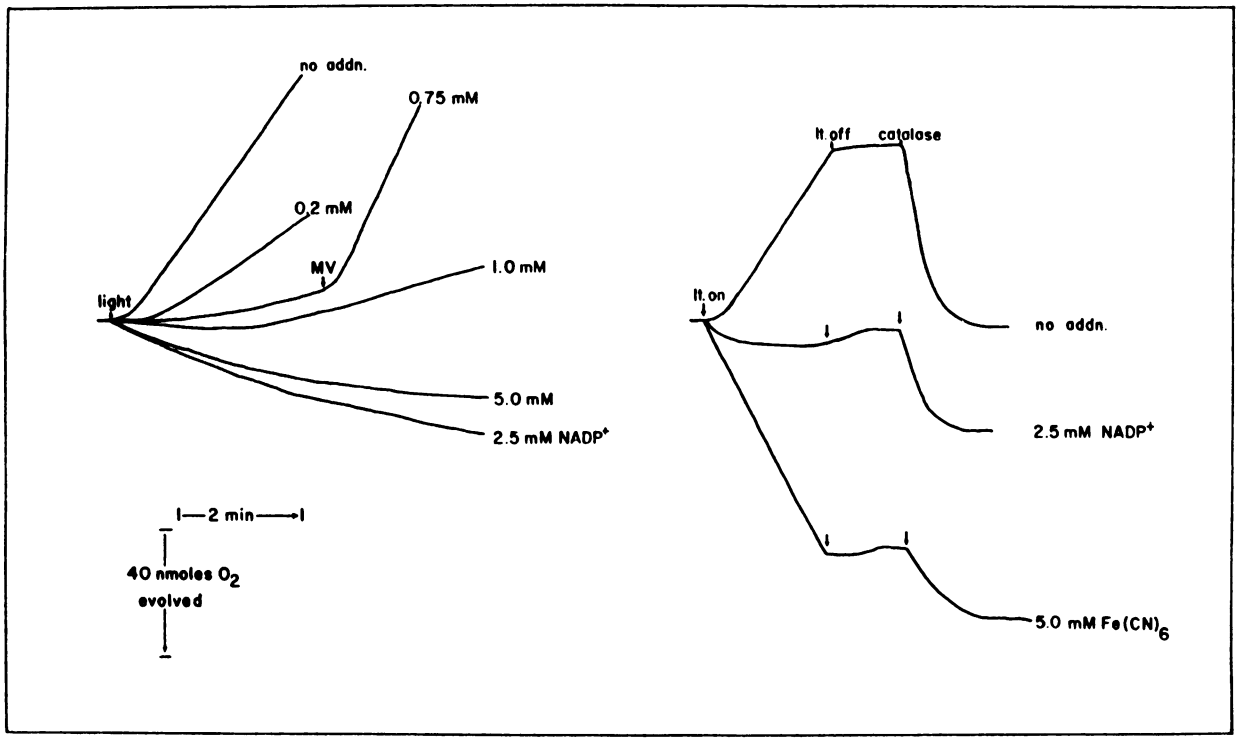

FIG. 6. Reduction of $NADP^+$ and of O_2 by EDTA/KCl-washed trichomes. The indicated amounts of $NADP^+$ were added to the reaction mixture and assayed for effects on the endogeous O_2 uptake reaction (left). The amount of H_2O_2 produced by the endogenous uptake reaction, in the presence or absence of added acceptors, can be monitored by dismutation of H_2O_2 when catalase is added after the period of illumination, as described in "Materials and Methods" (right).

Table IV. *The Effect of $MgCl_2$ upon $K_3Fe(CN)_6$- and $NADP^+$-Supported Photosynthetic Activities*

EDTA/KCl-washed trichomes were assayed with the following concentrations of added electron acceptors: 133 μM MV; 5.0 mM $K_3Fe(CN)_6$; 2.5 mM $NADP^+$. The reaction mixtures also contained 10 or 100 mM $MgCl_2$, where indicated, plus Hepes and NaCl as described in "Materials and Methods."

Acceptor	Assay	Activity	
		10 mM $MgCl_2$	100 mM $MgCl_2$
		μmol O_2 h^{-1} mg^{-1} Chl	
	H_2O endogenous[a]	106	57
MV	H_2O MV[a]	187	114
$Fe(CN)_6^{3-}$	O_2 evolution[a]	30	65
$Fe(CN)_6^{3-}$	H_2O endogenous[b]	80	14
$Fe(CN)_6^{3-}$	H_2O $Fe(CN)_6$[c]	110	79
	Total e^- transfer[c]	190	93
$NADP^+$	O_2 consumption[a]	91	33
$NADP^+$	H_2O endogenous[b]	114	38
$NADP^+$	H_2O $NADP^+$[d]	30	15
	Total e^- flow[c]	144	53

[a] Measured as change in O_2 concentration during illumination.
[b] Measured as O_2 evolution caused by the addition of catalase.
[c] Calculated from differences among directly measured values.
[d] Measured at 340 nm; corrected to O_2 equivalents.

The efficacy of this wash procedure is likely due to an effect on the external layer, or outer membrane, thus allowing lysozyme accessibility to the underlying peptidoglycan. There is evidence from other bacterial systems that the outer membrane is affected by EDTA treatment (19), and evidence from cyanobacteria that KCl shock releases periplasmic enzymes (25); thus, it is reasonable to conclude that the increased lysozyme sensitivity is due to an effective removal of the outer membrane.

Table V. *Cyclic Photophosphorylation Activity in EDTA/KCl-Washed and Sonicated Trichomes of S. platensis*

Photophosphorylation was conducted as described in "Materials and Methods." Samples assayed for phosphorylation were either washed trichomes, or washed trichomes that were subjected to a 2-min sonication prior to assay. Activities were corrected for a low level of background phosphorylation which was not light dependent.

Preparation	Photophosphorylation Activity
	μmol ATP h^{-1} mg^{-1} Chl
EDTA/KCl-washed	7
Washed, then sonicated	283

Spheroplasts of *Spirulina*, when stabilized by the presence of PEG, retain the capacity to carry out the Hill reaction with ferricyanide, but not CO_2 (Table III). Little dependence of this reaction on PD is seen, similar to the observation we report in Table III for the KCl/EDTA-washed trichomes. The presence of ferricyanide-dependent Hill activity in osmotically fragile unlysed spheroplasts presents a conundrum; integrity of these preparations necessarily involves retention of a membrane, and yet this same integrity allows access of ferricyanide, an anion, to sites of photoreduction within the spheroplasts. A tentative explanation for this observation is that the presence of a complex cell envelope in cyanobacteria has resulted in a functional separation of permeability properties, in which the outer membrane establishes the primary barrier to anionic permeation.

Trichomes of *Spirulina* permeabilized by the KCl/EDTA wash procedure possess a unique set of properties by which ionic strength and osmotic protection serve to regulate access of ferri-

cyanide to photoreduction sites. Thus, the wash procedure has left a permeability barrier to ferricyanide intact. Once overcome, ferricyanide reaches photoreduction sites near PSII; this reduction site is regulated by cations and we are currently examining this phenomenon to elucidate the mechanism of regulation.

At least one explanation for the cation effect might be that the EDTA/KCl wash procedure has caused extensive damage to the thylakoid membrane system of *S. platensis*, and thus these reactions are artifactual and therefore uninteresting. This is not a satisfactory explanation for such results because of the ability to demonstrate the photoreduction of exogenously added $NADP^+$ by these washed trichome preparations. This reaction requires Fd and Fd:NADP oxidoreductase, two components which can be solubilized from cyanobacterial membranes by mild perturbation (31). The fact that substantial rates of $NADP^+$ reduction can be observed indicates that even the most labile of photosynthetic electron transfer components are maintained during the EDTA/KCl washing procedure. The observation that ferricyanide is reduced by PSII rather than by PSI may be a reflection of a greater accessibility of the reducing side of PSII in cyanobacteria. A similar reduction of ferricyanide was observed in isolated thylakoids of *A. variabilis* and was shown to be due to a PSII-dependent reaction by DBMIB sensitivity and P/2e values (36). Here, perhaps the cyanobacterial system simply is different from that of the chloroplast. It can be similarly argued that the endogenous O_2 uptake reaction is not artifactual; it may simply be due to a forcing of the light reactions into the only pathway available in the absence of exogenous mediators and native Calvin cycle intermediates. There is firm evidence of an endogenous O_2 uptake reaction *in vitro* from mass spectrophotometric (26) and scopoletin fluorescence (24) data; whether this O_2 reduction is catalyzed by Fd or by Oxygen Reducing Substance (16) is unclear.

The data of Table V show that the EDTA/KCl-washed trichomes of *S. platensis* are impermeable to substrates of photophosphorylation; the impermeable species is probably ADP. When subjected to mild sonication, this permeability barrier is abolished, and photophosphorylation can be observed. These data indicate, as might be expected from our detection of $NADP^+$ photoreduction, that the energy-transducing capacity of the trichome thylakoids has not been damaged by the EDTA/KCl wash procedure. In this regard, we have achieved some success in attaining the goal stated by Myers (35): "it would be of obvious advantage to have a preparation of photosynthetic prokaryotic cells functionally analogous to chloroplasts of eukaryotes: a high fraction of original activity coupled to accessibility of additives." Thus, a balance has been achieved between preparative procedures that allow resolution of the system with concomitant accessibility of experimental probes, and procedures that allow preparations with high activities that are not artifacts of the preparative procedure.

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