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PHOTOSYNTHETIC PHOSPHORYLATION BY A MEMBRANE PREPARATION OF THE CYANOBACTERIUM *SPIRULINA MAXIMA*

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A new method for the isolation of photosynthetic membranes from the cyanobacterium *Spirulina maxima* has been developed. When illuminated, these membranes evolve oxygen in the presence of ferricyanide (Hill reaction) and consume oxygen in the presence of methyl viologen (Mehler reaction). When the membranes are left to stand at 4°C for 30 min, they develop the ability to consume oxygen in the light without an added, artificial electron acceptor. The Hill and Mehler reactions are not affected by the presence of ADP or uncouplers, but are inhibited by triphenyltin chloride. We have detected a cryptic ATPase activity stimulated by trypsin in the 20000×*g* supernatant fraction of the membrane preparation. In addition, the membrane vesicles contain an ATPase activity which is enhanced by treatment with dithiothreitol in the presence of light. These observations of ATPase led us to try a careful titration of the membrane vesicles with both triphenyltin chloride and *N,N'*-dicyclohexylcarbodiimide. When the vesicles were sealed with these reagents, we could observe both cyclic and stoichiometric photosynthetic phosphorylation.

Introduction

A limited number of papers concerning the photosynthetic phosphorylation activity of cyanobacterial membrane preparations have appeared [1–4]. The difficulties in observing photophosphorylation might be due to a greater susceptibility of these membranes during isolation to lose, extrinsic membrane proteins such as CF_1 that are essential for activity. Oligomycin at low concentrations [5] and F_1 coupling factor [6] enhance ATP synthesis in partially F_1-depleted submitochondrial particles. To explain these results, Racker and Horstman [6] suggested that the added coupling factor not only replaced missing enzymes but also facilitated the function of coupling factor still present in the particles. Thus, the coupling factor F_1 is seen to have both a structural and a catalytic role. The structural role could be successfully performed by oligomycin [5], DCCD [6] or F_1 lacking ATPase activity [7]. By titrating submitochondrial particles with oligomycin, Ernster and collaborators [5] established that the P/O ratio could be increased at low concentrations of this potent phosphorylation inhibitor. On this basis, we decided to look for substances with the same structural role described by Racker and Horstman [6] for F_1.

Gould [8] has reported that low levels of triphenyltin can restore the observed light-driven H^+ pumping in chloroplasts which have been partially depleted of coupling factors by EDTA treatment. Gould [8] found that triphenyltin did

Abbreviations: FCCP, carbonyl cyanide *p*-trifluromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine.

not inhibit electron transport as was the case with DCCD according to McCarty and Racker [9].

Thus, we applied these insights to cyanobacterial photophosphorylation. Membrane vesicles of *Spirulina maxima* were carefully titrated with triphenyltin to close H^+ channels which prevent the formation of the H^+ gradient needed to drive ATP synthesis.

The methods in the literature for the preparation of particles from cyanobacteria are gentle procedures. Lysozyme treatment of cells followed by osmotic shock to release membrane vesicles has been used [3]. However, this method is time consuming and yields small quantities of active particles. A method is described here for the preparation of membrane particles with good electron-transport activity in both photosystems as well as high rates of both cyclic and noncyclic photophosphorylation. These membrane preparations develop a very active endogenous oxygen-reducing system similar that described in other cyanobacteria [10].

Materials and Methods

S. maxima was collected with a Nytal nylon net (15 nm mesh) from the open culture of the Sosa Texcoco Co. at lake Texcoco, Mexico. The assays were carried out on the same day the cells were collected. Approx. 50 g wet weight of cyanobacteria were washed first by suspension in 100 ml of distilled water and filtered on the Nytal net. The cells were resuspended in 100 ml of distilled H_2O and sedimented by centrifugation at $500 \times g$ for 5 min at room temperature. Sometimes a large proportion of the cells did not sediment due to buoyancy conferred by gas vacuoles. When this occurred, the gas vacuoles within the cells were collapsed by pressing the cell suspension in a syringe. The sedimented cells were washed by centrifugation with 100 ml of homogenizing medium containing 0.5 M mannitol, 15 mM potassium phosphate buffer, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$ and 25 mM Tricine-NaOH pH 7.5. The cells were transferred to a mortar and 150 g of cold sand and 5 ml of the homogenizing medium were added. The cyanobacteria were ground gently with a pestle for 3 min at 4°C. 150 ml of homogenizing medium were mixed with the homogenate and this mixture was allowed to stand for 1 min to allow the sand to settle. The solution was decanted and centrifuged at $2000 \times g$ for 5 min at 4°C to remove sand, whole cells and large fragments.

The supernatant solution was centrifuged at $20000 \times g$ for 30 min at 4°C and the pellet was resuspended in 4 ml of cold homogenizing medium. The chlorophyll content was measured and then adjusted to 0.1 mg Chl/ml by addition of more homogenizing medium.

Oxygen production and consumption were measured with a Clark-type oxygen electrode (Yellow Springs Inc., Model 53 oxygen monitor) connected to a Heath Servo Recorder. Chlorophyll concentration was determined using the method of Arnon [11]. Illumination was done either with a Zeiss microscope lamp or a slide projector and light intensities are shown in Figs. 1 and 3.

Cyclic and stoichiometric photosynthetic phosphorylations were assayed by illuminating the samples of fresh particles for 1 min in a water bath at room temperature. The composition of the reaction medium was 0.5 M sucrose, 4 mM $MgCl_2$, 0.1% (w/v) albumin, 3 mM ADP, 4 mM potassium phosphate, 15 mM NaCl and 25 mM Tricine-NaOH, pH 8.0. The concentrations for a glucose-hexokinase trap were 25 mM and 25 U/ml respectively. The radioactivity was of the order of 300000 cpm ^{32}P for 1 μmol phosphate. The membrane preparation was added to give 10 μg Chl/ml and the final volume was 1 ml. The [γ-^{32}P]ATP synthesized was determined by measuring the esterification of ^{32}P to glucose, according to the method of Sone et al. [12].

ATPase activity was measured as described by Lien and Racker [13]. Protein content was estimated by the method of Lowry et al. [21].

P_i was determined by the method of Taussky and Shorr [14].

The methods used to activate and assay ATPase activity in the vesicles were the same as those of McCarty and Racker [15].

Results

Spinach chloroplasts require an auto-oxidizable electron acceptor such as methyl viologen for rapid photoreduction of oxygen, i.e., the Mehler reaction [16]. Honeycutt and Krogmann [10] had described

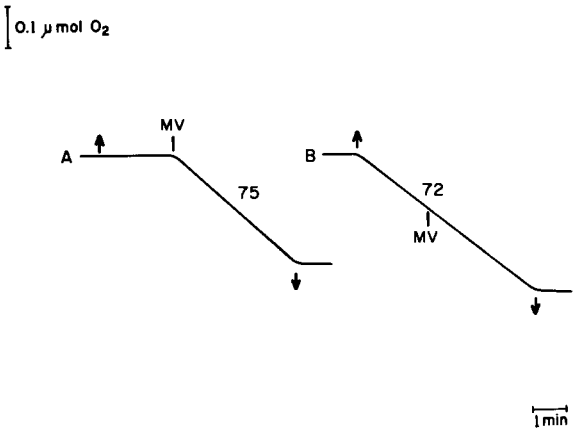

Fig. 1. Development of auto-oxidizable system in membrane vesicles. Electron transport was measured as oxygen consumption in a buffer containing mannitol as described in Materials and Methods. The samples were illuminated at an intensity of $1.6 \cdot 10^5$ erg/cm^2 per s. Membrane vesicles prepared from *S. maxima* were added to an amount of 12 μg/ml Chl. The temperature was 30°C. (A) Fresh membrane vesicles; (B) membrane vesicles incubated at 4°C for 30 min after their resuspension. Methyl viologen (MV) was added to a final concentration of $3.75 \cdot 10^{-4}$ M. The values shown are μmol O_2/mg Chl per h. Light on (↑), light off (↓).

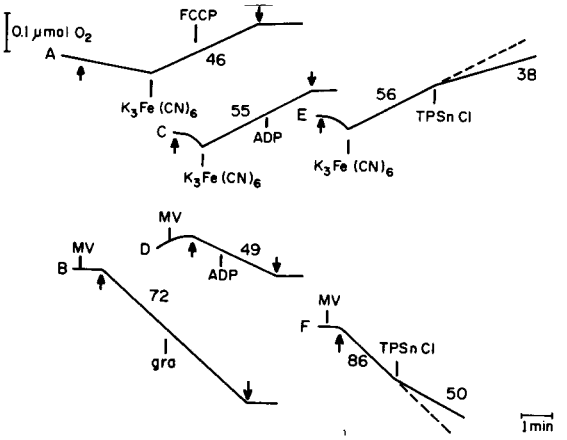

Fig. 2. The effects of ADP, uncouplers and triphenyltin chloride on photosynthetic electron transport. The medium, illumination, chlorophyll concentration and temperature were the same as in Fig. 1. The final concentrations of the reagents added were (A, C and E) $6.2 \cdot 10^{-4}$ M potassium ferricyanide, or (B, D and F) $3.75 \cdot 10^{-4}$ M methyl viologen (MV), (B) $1.2 \cdot 10^{-5}$ M gramicidin, (C and D) 1.2 mM ADP and (E and F) $3 \cdot 10^{-6}$ M tryphenyltin chloride (TPSnCl). (A) $6 \cdot 10^{-5}$ M FCCP. The values shown are μmol O_2/mg Chl per h. Arrows: same as in Fig. 1.

an oxygen-reducing system in the cyanobacterium *Anabaena variabilis*. We decided to examine *S. maxima* particles for cofactor requirements in this type of reaction. Fig. 1 shows that freshly prepared particles require methyl viologen for light-dependent oxygen consumption, but 30 min later this artificial electron acceptor is no longer needed. The experiment described in this figure is representative of ten experiments. Oxygen consumption with methyl viologen was always faster during a first illumination period than during subsequent exposures to light, so we worked only with particles exposed to light in a single measurement period. The concentration of $4 \cdot 10^{-4}$ M of methyl viologen was optimal for measurement of the Mehler reaction.

The experiments represented in Fig. 2 were done during 2 h, after preparing the particles. This figure shows the ability of chlorophyll-containing particles to carry out Hill and Mehler reactions in the presence of ferricyanide and methyl viologen, respectively. It is important to note that in the absence of $MgCl_2$ none of the above reactions take place, confirming the observation by Susor and Krogmann [17]. This figure also shows the failure of ADP, FCCP and gramicidin to induce any change in the rate of electron transport. Experiments with uncouplers were done in both Hill and Mehler reactions, but results from only one of these reactions are shown for each uncoupler in order to avoid repetition. Uncouplers were used at higher concentrations than those needed to stimulate the rate of electron transport in spinach chloroplasts [8]. Fig. 2 also shows the ability of triphenyltin chloride to inhibit oxygen production and consumption. These inhibition effects were maximal with $3 \cdot 10^{-6}$ M triphenyltin chloride and further additions did not enhance inhibition.

The inhibition of electron transport by triphenyltin, which has been reported as an H^+-channel blocker in the chloroplast ATPase complex [8], led us to examine the ATPase activities in both the supernatant and membrane vesicles. Indeed, there was a trypsin-activated ATPase activity in the supernatant of 27.5 nmol phosphate/mg protein per min, which indicates that some CF_1 has been detached from the membrane. However, an ATPase activity of 42 nmol phosphate/mg protein per min

remains associated with the membrane vesicles after light and dithiothreitol treatment. These activities cannot be regorously compared owing to the fact that different methods were used to activate each ATPase. It is interesting to note that some ATPase activity associated with the membrane vesicles is present prior to any activation (17.2 nmoles phosphate/mg protein per min).

After obtaining the above results, we decided to titrate the vesicles with triphenyltin chloride in a way similar to the experiments of Lee and Ernster [5] with submitochondrial particles. Our results are shown in Fig. 3 which describes the effects of triphenyltin on both stoichiometric and cyclic photosynthetic phosphorylations. This figure shows that the concentration of triphenyltin chloride for maximum activity in each phosphorylation reaction is distinct, as well as the concentration for maximum inhibition of electron transport. When stoichiometric photosynthetic phosphorylation was measured, the maximum value obtained was 170 μmol ATP/mg Chl per h in the presence of 10 nmol triphenyltin chloride and when cyclic photosynthetic phosphorylation was measured, the maximum yield was 721 μmol ATP/mg Chl per h in the presence of 1.5 nmol triphenyltin chloride.

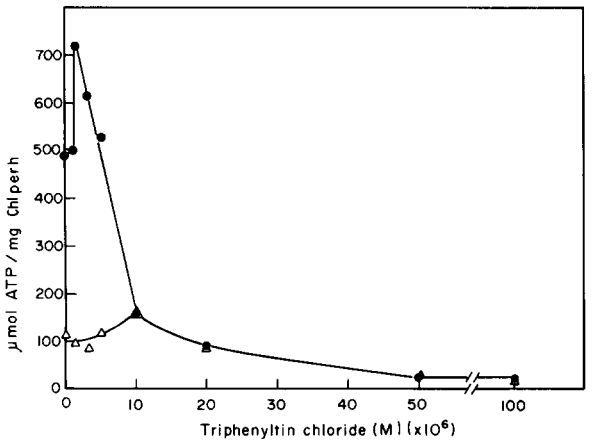

Fig. 3. Enhancing and inhibitory effects of triphenyltin chloride on cyclic and stoichiometric photosynthetic phosphorylation. Reaction conditions are described in Materials and Methods except for the addition of phenazine methosulfate to give a final concentration of 0.1 mM and methyl viologen (MV) up to 2.5 mM for cyclic and stoichiometric photosynthetic phosphorylation, respectively. Samples were illuminated for 1 min at room temperature at an intensity of $4.40 \cdot 10^5$ erg/cm^2 per s, ●——●, cyclic photophosphorylation; △——△, stoichiometric photophosphorylation.

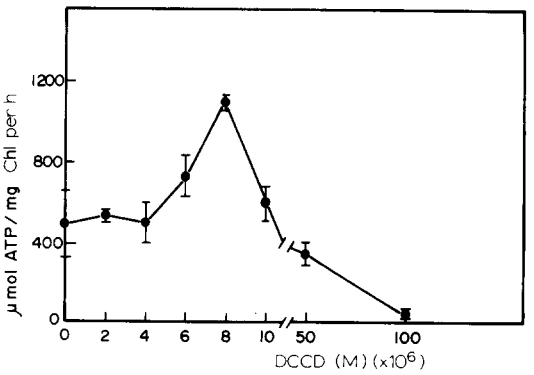

Fig. 4. Structural and inhibitory role of DCCD on cyclic photosynthetic phosphorylation. The reaction medium was the same as in Fig. 3. Membrane vesicles were incubated with DCCD for 10 min in the dark. (●) Average values calculated from four experiments. The bars represent the deviation from these results.

We decided to use another H^+-channel blocker to support our hypothesis that triphenyltin acts by blocking the H^+ channel. We tested DCCD and the results are presented in Fig. 4. Because DCCD inhibits chloroplast electron transport in the absence of ADP and phosphate, we only tested its

TABLE I

REQUIREMENTS FOR CYCLIC PHOTOSYNTHETIC PHOSPORYLATION

Temperature and reaction medium are described in Materials and Methods. Photosynthetic phosphorylation of the membrane vesicles was measured as the incorporation of $^{32}P_i$ into ADP and entrapped to glucose. 0.5 M sucrose was replaced by 0.25 M KCl in the preparation procedure.

Conditions	Incorporated radioactivity (cpm)	μmol ATP/mg Chl per h
Complete system	23747	921.6
−Glucose-hexokinase	16535	641.4
−Ascorbate-phenazine methosulfate	357.5	13.8
−ADP	241	9.3
−Light ($4.4 \cdot 10^5$ erg/cm^2 per s)	263	10.20
$-MgCl_2 + 1$ mM EDTA	306	11.4
−Membrane vesicles	100.5	3.6

effect on cyclic photosynthetic phosphorylation. After we found it takes 5 min for DCCD to inhibit photosynthetic phosphorylation (experiment not shown), *Spirulina* membrane vesicles were incubated in te dark with different concentrations of DCCD for 10 min. In this experiment the maximum value obtained for phosphorylation was 1130 μmol ATP/mg Chl per h in the presence of 8 nmol DCCD. We repeated the experiment at least four times.

Table I illustrates two important points: the requirements for the measurement of phosphorylation in membrane particles and the ability of vesicles which have been rapidly prepared in 0.25 M KCl rather than 0.5 M sucrose to achieve high rates of photophosphorylation in the absence of triphenyltin or DCCD.

Discussion

The best method reported thus far for the preparation of membrane vesicles of blue-green algae active in photosynthetic phosphorylation is lysozyme treatment of cyanobacteria followed by hypotonic disruption [2–4]. We tried this mild technique both in the presence and absence of EDTA [18]. After a long incubation time we had very low yields which would not permit the study of these membrane preparations. Hence, we tried disruption techniques such as sonication and the French press, but we found grinding with sand was the best method for obtaining good quantities of photosynthetically active membrane vesicles. The first property that drew our attention was the absence of the oxygen-reducing system in fresh membrane preparations, contrary to the results reported by Honeycutt [10] and Binder and collaborators [3].

Nevertheless, 30 min after resuspending the vesicles, an oxygen-reducing system appared with an electron flow from water, without an artificial electron donor. This peculiarity differs from the results of the above-mentioned authors [3,10]. The significance of the time elapsed until the autooxidizable factor's appearance remains to be established. However, we found some evidence to support the existence of proteolytic enzymes accompanying membrane preparations (data not shown). The oxygen-reducing substance might be generated by this enzyme's activity. Moreover, this substance was characterized only by Honeycutt and Krogmann [10] as a low molecular weight peptide, and further studies remain to be done in order to answer this question.

Oxygen-production (chloroplasts) [8] or -consumption (mitochondria) [20] rates can increase when ADP or uncouplers are added to the reaction medium. This kind of increment is good evidence to support the existence of a coupled state between electron transport and ATP synthesis. However, the absence of any increment does not directly imply an uncoupling [19]. One of the reasons for the lack of this kind of increment in membrane preparations is the release of coupling factor CF_1 from the membrane. If there is no increment after the addition of ADP or uncouplers, this does not imply an intrinsic low activity of ATP synthesis coupled to the open electron-transport chain in cyanobacteria. The absence of increments would rather be due to the ease with which ATPase is detached from the membrane when the vesicles are prepared.

The presence of sufficient CF_1 attached to the membrane vesicle was referred to in Results. The basal ATPase activity of chlorophyll-containing membranes is 40% of the total. This basal activity obtained without treatment or illumination in the presence of dithiothreitol can be attributed once more to the presence of proteolytic enzymes in the preparation. This question is open to discussion.

Our membrane vesicles were able to present a good rate of cyclic as well as stoichiometric photosynthetic phosphorylation due to a careful titration of the vesicles with triphenyltin chloride.

It is not clear why the amount of triphenyltin chloride required to produce a transition in the electron-transport rate (Fig. 2) is smaller than that necessary to attain the stoichiometric photophosphorylation maximum. (Fig. 3). These differences could be due to the fact that different preparations were used for the two experiments. Another reason to explain these results would be different conditions in both measurements. This difference in affinity could be the result of conformational changes occurring in the membrane energized by light. Experiments are in progress to clarify this point. Enhancement and inhibition effects of triphenyltin on the photophosphorylation of

Spirulina indicate that this compound is able to block the H^+ channel of the cyanobacteria in a way similar to that occurring in chloroplasts [8]. Moreover, this is the first time that the effect of triphenyltin chloride has been studied in cyanobacteria. Further studies remain to be done in order to measure the H^+ accumulation in chlorophyll-containing membrane vesicles and to understand the role of the basal ATPase activity in this preparation.

At low concentrations, DCCD (Fig. 4) enhances photosynthetic phosphorylation and is a potent inhibitor at high concentrations, as expected.

Binder and co-workers [3] have reported that a good rate of ATP synthesis can be preserved when the membrane particles are prepared in a ionic solution, particularly with K^+. We were able to confirm this result by preparing *S. maxima* membrane vesicles in the presence of 0.25 M KCl (Table I). The basal ATPase activity present in the membranes was overcome by using a glucose-hexokinase trap.

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