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ROLE OF DIVALENT CATIONS AND ASCORBATE IN PHOTOCHEMICAL ACTIVITIES OF *ANACYSTIS* MEMBRANES

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Summary

Photosynthetic membrane fragments were prepared from *Anacystis nidulans* by French pressure cell disruption. Ascorbate was required to stabilize photophosphorylation activity in membranes kept at near 0°C. Divalent cations were required during mechanical disruption and during assays for Photosystem II activity, with Ca^{2+} serving best. The rate of photophosphorylation was severely inhibited by Ca^{2+} during assays. Results suggest that best rates are achieved when photosynthetic membranes contain Ca^{2+} exposed to the interior surface, facilitating Photosystem II activity, and Mg^{2+} exposed to the exterior surface during assays, facilitating photophosphorylation activity.

Relatively few studies of photosynthetic light reactions have utilized isolated membranes from blue-green algae, due in part to the difficulty in obtaining membranes which retain all photochemical activities. Photosystem II and noncyclic photophosphorylation require careful control of osmotic support during all stages of the preparation, and divalent cations during the assays [1]. The presence of divalent cations during cell disruption also improves activity [2]. Mechanical disruption of intact cells destroys Photosystem II activities [3], so cell walls are usually removed by lysozyme digestion Exposure of the isolated membranes from *Anacystis nidulans* to low temperatures impairs photophosphorylation [4], requiring membrane preparation at room temperature.

Recently, membranes which retained Photosystem II activities when assayed in the presence of Ca^{2+} were prepared from several blue-green algae

Abbreviations: Chl, chlorophyll,; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMPD, *N*, *N*, *N'*,*N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

by French pressure cell disruption [5]. Membranes from *Anacystis* retained high Photosystem II activities only when cells were broken, and isolated membranes were assayed, in the presence of Ca^{2+} [6]. Here we describe methods whereby membranes which retain all photochemical activities can be prepared from *Anacystis* rapidly.

Anacystis nidulans TX 20[7] was grown in continuous culture at 39°C or 25°C as described previously [8]. Cells were pelleted by centrifugation, and resuspended in medium containing 0.1 M Tricine-NaOH (pH 7.5), 0.4 M sucrose, 0.05 M $CaCl_2$ and 0.0075 $g \cdot ml^{-1}$ Ficoll. When no Photosystem II assay was to be performed, Ca^{2+} was deleted from the medium. Resuspended cells (0.15 mg Chl$\cdot ml^{-1}$) were passed through a French pressure cell (20 000 lb/inch^2, 50 ml$\cdot min^{-1}$). Broken cells were centrifuged at 6 000 × *g* for 5 min. Supernatant was centrifuged at 20 000 × *g* for 20 min. The pellet, containing membrane fragments and some wall material, was resuspended in medium containing 0.05 M Tricine - NaOH (pH 7.3) and 0.4 M sucrose. Chl concentration was 0.15 mg$\cdot ml^{-1}$. Membrane fragments were maintained in dim light prior to performing assays. Preparations were at room temperature when photophosphorylation assays were to be performed; otherwise preparations were at near 0°C.

Electron transport assays were performed as described previously [6]. Photophosphorylation assays measured the rate of incorporation of $H^{32}PO_4^{2-}$ into ATP [9] at 25°C. Cyclic photophosphorylation assays (3 ml volume) contained, in μmol: 200 Tricine-NaOH (pH 7.6), 36 $MgCl_2$, 15 ADP, 30 P_i (containing 1μCi $H^{32}PO_4^{2-}$), 0.1 phenazine methosulfate, 100 sodium ascorbate. Membrane fragments, containing 10 mg Chl$\cdot ml^{-1}$, were added last to assays. For noncyclic photophosphorylation assays 1 μmol $K_3Fe(CN)_6$ was present instead of the phenazine methosulfate and ascorbate. Assays were stopped by adding 0.3 ml of 20% trichloroacetic acid. ATP was extracted and analyzed as described previously [10].

Chl concentration was determined spectrophotometrically [11]. All chemicals were reagent grade.

It was desirable to prepare and incubate membrane fragments at 0°C, since this stabilized electron transport activities longer than at room temperature. However, photophosphorylation activity was considerably diminished in chilled membranes (Fig. 1, compare open circles with open triangles). When ascorbate was present during low-temperature incubation, very little activity was lost for several h at either temperature (Fig. 1, closed symbols).

The results shown in Fig. 1 were from membranes derived from cells grown at 39°C. Virtually identical results were obtained in preparations from cells grown at 25°C. When ascorbate was withheld until immediately prior to assay it slightly stimulated activity independent of incubation temperature, but did not restore the activity lost with time at 0°C. No reagent tested, including dithiothreitol and the chloride and nitrate salts of K^+, Na^+, Ca^{2+} and Mg^{2+} would substitute for ascorbate in preventing chilled membranes from losing photophosphorylation activity.

Virtually identical results were obtained when the activity of noncyclic photophosphorylation accompanying ferricyanide reduction was measured, except absolute rates were approx. 35% of those shown in Fig. 1. However,

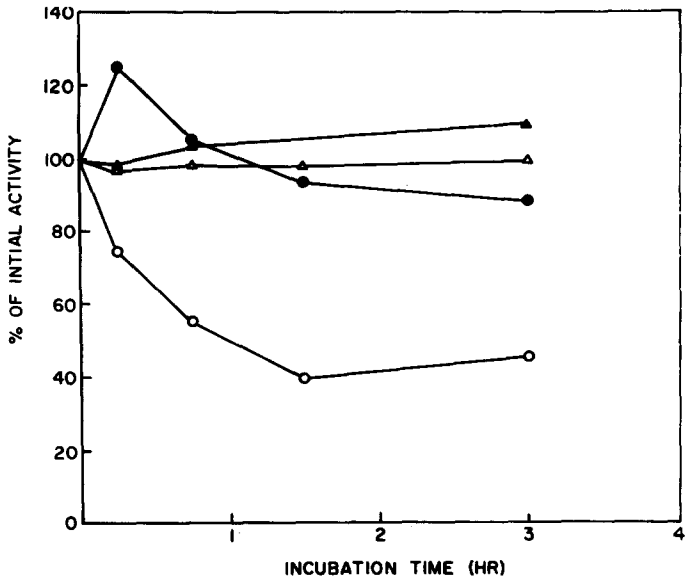

Fig. 1. Effect of preincubation conditions on stability of cyclic photophosphorylation in *Anacystis* membranes. Membrane fragments were prepared as described, except the final resuspension medium contained 0.1 M sodium ascorbate where indicated. Incubation conditions were: △—△, 25°C minus ascorbate; ○—○, 0°C minus ascorbate; ▲—▲, 25°C plus ascorbate; ●—●, 0°C plus ascorbate. Activities at zero time were 450 μmol ATP·mg^{-1} Chl·h^{-1}.

for these measurements membranes had to be pelleted and resuspended in medium devoid of ascorbate immediately prior to essays.

It has been proposed previously that chill damage in *Anacystis nidulans* results from a direct effect on photophosphorylation [4]. Then one might expect that membranes from cells resistant to chilling (those grown at 25°C) would not lose photophosphorylation activity subsequent to chilling, while membranes from cells sensitive to chilling (those grown at 39°C) [8] would lose activity. However, membranes from cells grown at either temperature had diminished photophosphorylation activity subsequent to chilling, unless ascorbate was present during cold incubation. Therefore, the mechanism of chill damage in *Anacystis* is not apparently correlated with damage to the photophosphorylation mechanism. Neither did chilling affect electron transport activities in isolated membranes from cells grown at either temperature.

As previously shown [6], Photosystem II activities were highly dependent on the presence of Ca^{2+} during cell disruption and during assays. Mg^{2+}, Mn^{2+} and Sr^{2+} each substituted partially for Ca^{2+}, but rates were approx. 40% as high. Photosystem I activity (measured as electron transfer from ascorbate/TMPD to methyl viologen in the presence of DCMU [12]) was unaffected by divalent cations.

When $CaCl_2$ (0.05 M) was present during French pressure cell disruption, the rate of cyclic photophosphorylation was diminished by approx. 20% over control rate with no added divalent cation (control rate = 450 μmol ATP·mg^{-1} Chl·h^{-1}), even when the $CaCl_2$ was removed from the membrane fraction immediately after preparation. The presence of other divalent cations

during disruption had little effect on photophosphorylation. Noncyclic photophosphorylation rates were significantly higher in membranes from cells broken in the presence of divalent cations. Ca^{2+} worked best, giving double the rate obtained when no divalent cations were present. (Rate with 0.05 M $CaCl_2$ = 150 μmol ATP·mg^{-1} Chl·h^{-1})

Ca^{2+} significantly inhibited photophosphorylation when present in the concentration which stimulates Photosystem II maximally (Table I). No concentration of Ca^{2+}, when present during photophosphorylation, stimulated activity. Therefore, Ca^{2+} does not appear to play a direct role in ATP formation during photophosphorylation assays, but effectively competes with Mg^{2+}.

A summary of the effects of divalent cations on photochemical activities as presented in this and the previous paper [6] is shown in Table II. These data can be explained by assuming that isolated membrane vesicles from *Anacystis* have a site exposed on the interior surface associated with Photosystem II which requires Ca^{2+} for most efficient function, while the coupling factor which requires Mg^{2+} is exposed to the exterior surface. Photosystem I does not require either site. Noncyclic photophosphorylation requires Ca^{2+} during cell disruption in order to trap it on the inside of vesicles formed during French pressure cell disruption; thereby facilitating electron transport; Mg^{2+} is required during assays to support photophosphorylation.

The hypothesis that Ca^{2+} acts only in the inner side of vesicles formed during cell disruption is supported by the observation that pelleting and re-

TABLE I

EFFECT OF Ca^{2+} AND Mg^{2+} DURING ASSAYS ON CYCLIC AND NONCYCLIC PHOTOPHOSPHORYLATION
Ca^{2+} and/or Mg^{2+} were added where indicated as the chloride salt at 0.01 M.

Divalent cation added	μmol ATP·mg^{-1} Chl·h^{-1}	
	Cyclic	Noncyclic
None	152	18
Ca^{2+}	84	0
Mg^{2+}	485	125
Ca^{2+} and Mg^{2+}	162	0

TABLE II

DIVALENT CATION REQUIREMENT FOR PHOTOSYNTHETIC ACTIVITIES IN *ANACYSTIS*
Photosystem I (PS I) = ascorbate/TMPD to methyl viologen, Photosystem II (PS II) = H_2O to $Fe(CN)_6^{3-}$ or benzoquinone, cyclic photophosphorylation (PHP) = phenazine methosulfate mediated, noncyclic PHP = coupled to H_2O to $Fe(CN)_6^{3-}$.

Assay	Requirement during	
	cell disruption	photochemical assay
PS I	none	none
PS II	Ca^{2+} (Mg^{2+}, Mn^{2+}, Sr^{2+} poorly)	Ca^{2+} (Mg^{2+}, Mn^{2+}, Sr^{2+} poorly)
Cyclic PHP	none	Mg^{2+}
Noncyclic PHP	Ca^{2+}	Mg^{2+}

suspending the membranes in medium devoid of Ca^{2+} does not decrease activity as long as Ca^{2+} is present during cell disruption, when the membrane vesicles are formed. This interpretation is strengthened by the demonstration that cells broken in medium devoid of Ca^{2+} (thereby retaining less than 25% of the Photosystem II activity obtained when Ca^{2+} is present) regain over 60% of the activity when the membrane fraction is passed a second time through the French pressure cell, this time in the presence of 0.05 M Ca^{2+}.

In summary, when suitable medium is used for cell disruption and assays, membrane vesicles form *Anacystis nidulans* can be obtained rapidly, which retain all of the photochemical activities associated with the light reactions of photosynthesis.

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