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RECONSTITUTION OF CYANOBACTERIAL PHOTOPHOSPHORYLATION BY A LATENT Ca^{+2}-ATPase

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Summary: Photosynthetic membranes derived from sonic extracts of the cyanobacterium Spirulina platensis contain a latent Ca^{+2}-ATPase which is activated by exposure to trypsin. When sonic membranes are washed with ethylenediaminetetraacetic acid, the ATPase is removed from these membranes with an accompanying loss of photophosphorylation activity. The latent ATPase activity solubilized by washing has been partially purified, and addition of the enzyme to depleted membranes restores photophosphorylation activity to levels approaching 50% of the rates observed in unwashed membranes. These data indicate that this ATPase is the coupling factor responsible for photosynthetic energy transduction in Spirulina platensis.

Introduction: Reconstitution of phosphorylation coupled to electron transport has been shown to involve readdition of coupling factors to depleted membranes derived from mitochondria, chloroplasts and bacteria. These high molecular weight enzymes, such as that derived from chloroplasts, consist of up to five non-identical subunits (1), and exhibit ATPase activity in the presence of either Ca^{+2} or Mg^{+2} (2). The ATPase activity of the chloroplast enzyme is latent, and can be activated by trypsin, heat, or, for the membrane-bound enzyme, by illumination in the presence of thiols (3).

Little is known about the coupling apparatus in cyanobacteria. Petrack and Lipmann (4) demonstrated the existence of a light-activated ATPase in Anabaena variabilis, and Biggins (5) and Lee et al. (6) isolated proteinaceous "phosphorylation factors" from cyanobacteria. Recently, Binder and Bachofen (7) reported the purification of a latent Ca^{+2}-ATPase from a thermophilic cyanobacterium; however, this enzyme did not reconstitute photophosphorylation activity. In this communication, we report the isolation and partial purifi-

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cation of a latent Ca^{+2}-ATPase from the cyanobacterium Spirulina platensis, and present data to indicate that this enzyme is a cyanobacterial coupling factor.

Materials and Methods: Axenic cultures of Spirulina platensis were grown at 35° under illumination provided by a bank of fluorescent lights using the growth medium of Ogawa and Terui (8). Late log-phase cells were harvested by centrifugation, washed once with 20 mM Tricine (pH 8) containing 10 mM $MgCl_2$, and resuspended in this medium to a final concentration of 0.1 mg chl[1]a/ml. These, and all subsequent operations, were carried out at 4° unless otherwise noted. The cells were sonicated in 150 ml batches at full output with a Branson W-185 sonicator for 5 min. in a salt-ice bath. The resulting sonicate was centrifuged (2,500xg, 10 min.) to remove debris, and the supernatant was then centrifuged (39,000xg, 1 hr.) to harvest chl-containing membranes. These phosphorylating particles were either stored at -70° in STN buffer containing 2 mg/ml bovine serum albumin or diluted to 50 μg chla/ml in 2 mM Tricine (pH 8) containing 1 mM EDTA. This suspension was stirred (30 min.) and centrifuged (20,000xg, 30 min.). The pellets were subjected to a second EDTA wash, centrifuged, and the resulting non-phosphorylating particles were stored at -70° in STN buffer.

For extraction of the latent Ca^{+2}-ATPase, whole cells (50 mg chla) were washed twice with 200 ml of 10 mM Tricine (pH 8) containing 10 mM EDTA and 5% polyethyleneglycol, resuspended in 200 ml of 10 mM Tricine (pH 8) containing 5 mM EDTA, 10% polyethyleneglycol and 1.5 M KCl, and homogenized for 1 min. at top speed in a Waring blendor. The cells were centrifuged (10,000xg, 10 min.), resuspended in 20 mM Tricine (pH 8) containing 10 mM $MgCl_2$ to a final concentration of 0.1 mg chla/ml, and sonicated for 5 min. as described above. After removal of cell debris, protamine sulfate (2 mg/mg chla) was added to the supernatant with stirring, and the suspension was permitted to stir for 30 min. Centrifugation (10,000xg, 20 min.) yielded a pellet containing nearly all of the chl-containing membranes. These pellets were washed twice (at 25°, otherwise as described above) with Tricine-EDTA to solubilize the Ca^{+2}-ATPase activity. The two washes were pooled, brought to final concentrations of 20 mM Tris-SO_4(pH 7.2), 1 mM ATP, and 30% $(NH_4)_2SO_4$. After centrifugation (10,000xg, 10 min.) to remove chl-containing material, the supernatant was brought to 65% saturation with solid $(NH_4)_2SO_4$ and cooled to 4°. The resulting precipitate contains the crude ATPase.

Latent ATPase activity was partially purified by dissolving the pellets from 300 ml of the centrifuged extract in 100 ml of buffer containing 0.18 M Tris-Cl (pH 7.8), 1 mM EDTA and 1 mM ATP. This solution was applied to a DEAE-cellulose column (2.5x15 cm) equilibrated with the same buffer. After loading, the column was washed with 50 ml of 0.25 M Tris-Cl containing EDTA and ATP, and the ATPase was then eluted from the column by raising the Tris concentration to 0.4 M. The pooled protein-containing fractions (about 50 ml) were warmed to 20° and brought to 65% saturation by addition of solid $(NH_4)_2SO_4$, and the resulting suspension was stored at 4° until assay.

Protein was estimated by the method of Lowry, et al.(9) using bovine serum albumin as the standard, and MacKinney's method (10) was used for chla. Photophosphorylation assays using PMS at the concentrations specified in "Results" were conducted in a reaction mixture which contained 50 mM Tricine (pH 8), 50 mM NaCl, 1 mM ADP, 5 mM $NaH_2{}^{32}PO_4$ (10^6 CPM/ml), and 3 mM $MgCl_2$. After 2 min. of white light illumination (10^6 ergs/cm^2.sec) samples were acidified with trichloroacetic acid and ATP synthesis was estimated by gas-flow counting after

[1]Abbreviations: chl, chlorophyll; STN, 0.4 M sucrose-20 mM Tricine (pH 8)-15 mM NaCl; PMS, phenazinemethosulfate; SDS, sodium dodecyl sulfate.

extraction of unreacted phosphate by the method of Avron (11). Assays of ATPase activity were carried out according to the method of Lien and Racker (3), with modified concentrations of trypsin as described in "Results". All coupling factor and ATPase assays were conducted on protein which was desalted by passage through a G-25 Sephadex column (1x30 cm) equilibrated with 20 mM Tricine, 1 mM EDTA. Acrylamide gel electrophoresis was conducted using 0.6x7 cm gels and the buffer system described by Laemmli (12). The buffers, bovine serum albumin, ATP, ADP and reagents for gel electrophoresis were obtained from Sigma. All other chemicals used were of the purest grades commercially available.

Results: Table I presents data which indicate that when sonic membranes from Spirulina are exposed to high concentrations of trypsin, Ca^{+2}-ATPase activity is elicited; no activity is produced in these preparations by heat treatment (60°) following established procedures (3). Table II shows that Ca^{+2}-ATPase activity is depleted from sonic membranes by exposure to EDTA, and that the decrease in this ATPase activity is accompanied by loss of photophosphorylation activity. Using the protocols described in "Materials and Methods", we have partially purified the latent Ca^{+2}-ATPase from EDTA washes. The results of this procedure, summarized in Table III, indicate that a 20-fold purification of enzymatic activity has been achieved.

Table I. Activation of membrane-bound Ca^{+2}-ATPase activity by trypsin.

Trypsin concentration (μg) in activation mixture*	ATPase activity (μmoles phosphate/hr·mg protein)
0	0.59
40	3.25
400	5.06

*Membranes were incubated for 30 min. with the indicated trypsin concentrations before transfer to the ATPase reaction mixture.

Table II. Effect of EDTA washing on the activity of Spirulina membranes.

Treatment	Photophosphorylation[1]	Ca^{+2}-ATPase[2]
None	631	310
Twice EDTA Washed	0	113

[1]μmoles ATP/hr·mg chl
[2]μmoles phosphate released/hr·mg protein

Table III. Partial purification of Ca^{+2}-ATPase activity from Spirulina.

Fraction	Protein (mg)	Activity (units*)	Sp. Activity (units/mg)	Yield (%)
Crude Extract	6,600	16,040	2.4	100
EDTA Supernatant	1,460	10,610	7.3	66
$(NH_4)_2SO_4$ Precipitate	554	4,280	7.9	27
DEAE Eluate	67	2,600	38.7	16

*one unit is defined as 1 μmole of phosphate released per hour.

Preliminary experiments with EDTA-washed membranes indicated that both crude EDTA extracts and the partially purified Ca^{+2}-ATPase would reconstitute photophosphorylation activity in the washed membranes. We therefore sought to determine the optimal conditions for reconstitution, using the partially purified enzyme. Figure 1 shows the effects of $MgCl_2$ concentration and of time of incubation of the enzyme with washed membranes on reconstitution of ATP synthesis; these data suggest that optimal reconstitution requires 30 mM $MgCl_2$ combined with a 30 min. incubation period. Using these conditions, we next examined the extent of reconstitution with varying concentrations of protein (Fig. 2); these data indicate that substantial reconstitution of photophosphorylation activity (nearly 50% of the activity observed in unwashed particles) can be achieved with the partially purified enzyme.

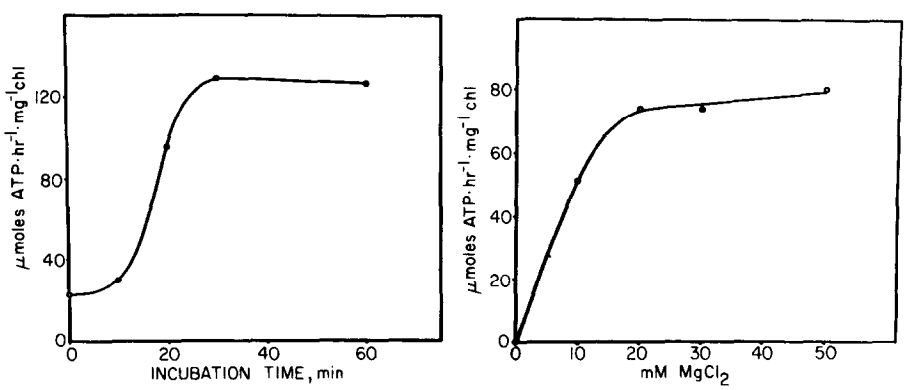

Figure 1. Effects of incubation time (enzyme plus membranes, left) and $MgCl_2$ concentration (right) on reconstitution of photophosphorylation. Membranes (50 μg chla) and enzyme (375 μg protein) were incubated in 0.5 ml of 20 mM Tricine (pH 8) plus 1 mM EDTA for 30 min. (left) or in the presence of 20 mM $MgCl_2$ (right). Aliquots (200 μl) were transferred to reaction mixtures containing 66 μM PMS for assay of activity.

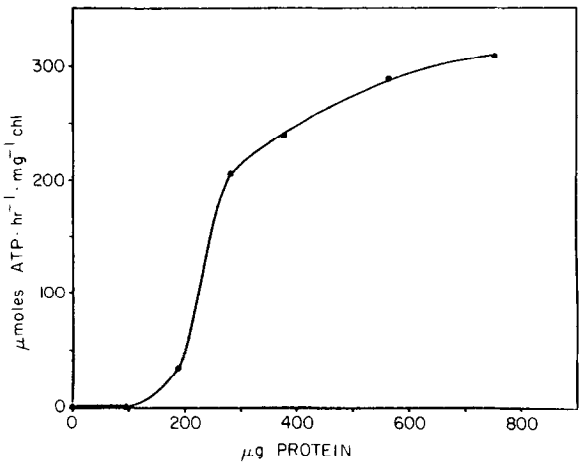

Figure 2. Effect of coupling factor concentration on reconstitution of photophosphorylation. Membranes (25 μg chla) were incubated with the amounts of protein indicated for 30 min. in the presence of 30 mM $MgCl_2$, 20 mM Tricine (pH 8) and 1 mM EDTA. Aliquots (100 μl) were transferred to reaction mixtures containing 125 μM PMS for assay of activity.

In order to assess the purity of the Spirulina coupling factor, acrylamide gel electrophoresis was performed in the presence of SDS (Fig. 3). It is apparent from this experiment that the coupling enzyme is not pure. In addition to the bands indicated by arrows, which electrophorese in positions comparable to those observed with the subunits of the chloroplast enzyme (1), several high- and low-molecular weight impurities are visible.

Discussion: These data represent the first reported reconstitution of photophosphorylation in cyanobacterial membranes by an enzyme of defined activity (Ca^{+2}-ATPase). Although the coupling factor has not yet been purified to homogeneity, it has several interesting properties. Foremost among these is the persistent latency of the enzyme; neither heat nor low concentrations of

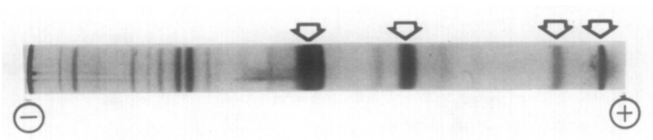

Figure 3. SDS-Gel electrophoresis of the partially purified coupling factor. Protein (30 μg) was denatured and electrophoresed on a 7.5% gel. Arrows indicate the bands migrating at positions similar to the subunits from the chloroplast enzyme.

trypsin are sufficient to elicit measurable amounts of Ca^{+2}-ATPase activity. Although digestion with high concentrations of trypsin activates the Ca^{+2}-ATPase, we cannot be certain that that this procedure generates optimal rates of ATP hydrolysis; it is quite possible that extensive trypsin digestion destroys, as well as activates the enzyme. Secondly, the enzyme exhibits a sensitivity to room temperature conditions (data not shown) which has prevented us from carrying out purification along the lines established for the spinach enzyme (3). We have been able to partially purify a reconstitutively active enzyme only by carrying out chromatographic procedures at 4^{o}. Third, although this coupling factor exhibits an unexpected instability to room temperature conditions, the enzyme isolated by our procedure at 4^{o} shows exceptional reconstitution activity, restoring rates of ATP synthesis in EDTA-washed membranes to levels approaching 50% of the rates observed in unwashed membranes.

Until the Spirulina coupling enzyme is purified to homogeneity, it will not be possible to assign specific functions to the major protein bands resolved by electrophoresis on SDS gels. Failure to observe any heat-activated ATPase activity with this enzyme, and our inability to purify a reconstitutively active preparation at room temperature suggests that the Spirulina coupling factor is uniquely sensitive to temperature. We cannot determine at present whether this sensitivity is manifested by the loss of one or more essential subunits from the enzyme.

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