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SITE-SPECIFIC INACTIVATION OF THE PHOTOPHOSPHORYLATION REACTIONS OF *ANABAENA VARIABILIS**

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SUMMARY

The photophosphorylation activities of chlorophyll-containing particles from *Anabaena variabilis* reflect the method of cell disruption. Lysozyme treatment followed by osmotic shock and differential centrifugation yields particle preparations active in both stoichiometric and cyclic phosphorylation. Sonic or mechanical disruption of the cells in 0.4 M sucrose gives particle preparations which are active only in cyclic phosphorylation. Further sonication of these particles in absence of sucrose removes protein essential for cyclic phosphorylation. Cyclic phosphorylation activity can be restored to the depleted particles by readdition of the extracted protein. The depleted particles can catalyze the light-dependent transfer of electrons from reduced indophenol dye to $NADP^+$ only when supplemented with either plastocyanin or cytochrome C_{554}, and the protein required for cyclic photophosphorylation has no effect on this activity. Conversely, plastocyanin and cytochrome C_{554} have no effect on the restoration of cyclic photophosphorylation.

INTRODUCTION

In vitro photosynthetic phosphorylation accompanies the stoichiometric transfer of electrons from water to $NADP^+$ or other suitable oxidants. Phosphorylation may also be elicited by a cycling of electrons in the presence of an added redox catalyst such as phenazine methosulfate (PMS). There are many indications in the literature that these reactions may involve different or multiple sites of ATP synthesis. Work with blue-green algae gives new evidence to the notion of multiple phosphorylation sites. Both BIGGINS[1] and GERHARDT AND TREBST[2] have reported on the cell-free activities of blue-green algae following dissolution of the cell wall by lysozyme. These preparations are active in the photophosphorylation which accompanies the reduction of $NADP^+$ or ferricyanide, as well as the cyclic phosphorylation elicited by PMS. In contrast, chlorophyll-containing particles prepared from sonic or mechanically disrupted cells show only cyclic phosphorylation activity[3]. We have compared the two preparative procedures and wish to emphasize the greater stability of the cyclic phosphorylation reaction when compared to the stoichiometric one. The stoichiometric phosphorylation activity of lysozyme-prepared material is easily and irrever-

Abbreviation: PMS, phenazine methosulfate.

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sibly lost by treatments which disrupt particle structure. The more stable cyclic phosphorylation can be reversibly inactivated by extraction of a protein constituent, as first shown by BIGGINS[1]. Our extraction experiments suggest that plastocyanin is not a participant in the cyclic phosphorylation process.

MATERIALS AND METHODS

The procedures for growth of *A. variabilis*, isolation of chlorophyll-containing particles and storage of these preparations have already been reported[4], as have the methods for measurement of cyclic phosphorylation[3]. We have previously described the methods for assaying $NADP^+$ reduction and for the purification of ferredoxin, cytochrome C_{554} and plastocyanin[5,6]. Lysozyme digestion of algal cell walls was accomplished by suspending 15 g of freshly harvested packed cells in 100 ml of 0.4 M sucrose–0.03 M phosphate buffer (pH 6.8) to which 50 mg of egg white lysozyme was added. The cell suspension was incubated with stirring for 18 h at 2°, then the cells were washed several times with 0.4 M sucrose to remove the lysozyme, since several commercial preparations of lysozyme contained inhibitors of the photosynthetic activities. The treated cells were then resuspended in distilled water to lyse the protoplasts. Solid sucrose was promptly added to give a concentration of 0.4 M so as to minimize exposure to low osmolarity and prevent further loss of activity. About 25 % of the cells are lysed under these conditions. The suspension was centrifuged at 8000 × *g* for 10 min to remove unbroken cells and large debris. The resulting supernatant was then centrifuged at 108000 × *g* for 1 h to concentrate the chlorophyll-containing particles. The particles were resuspended in 0.4 M sucrose solution and used immediately.

RESULTS

Table I shows a comparison of the photosynthetic activities of chlorophyll-containing particles from cells disrupted by enzymatic lysis, sonication and mechanical rupture with glass beads. The results are representative of measurements on a minimum of 20 preparations by each method. All preparations show a high rate of $NADP^+$ reduction and of PMS-induced cyclic phosphorylation. Only the particles prepared by lysozyme digestion show phosphorylation accompanying $NADP^+$ reduction. The lower half of Table I shows the unique lability of the NADP stoichiometric phosphorylation relative to the other photosynthetic activities. When lysozyme-prepared material was diluted to a concentration of 0.1 mg chlorophyll per ml in distilled water and the various activities promptly measured, PMS-induced cyclic phosphorylation and $NADP^+$ reduction were hardly affected, while about half of the NADP phosphorylation activity was lost. Brief sonication of lysozyme-prepared material destroys 80 % of the phosphorylation accompanying $NADP^+$ reduction while causing only a 36 % loss in the rates of $NADP^+$ reduction or PMS cyclic phosphorylation. As yet, we have found no way to restore the NADP phosphorylation activity of these damaged preparations.

BIGGINS has reported on the reversible removal of a protein necessary for cyclic phosphorylation by preparations from the blue-green alga, Phormidium[1]. We have confirmed this observation with *A. variabilis*, and the data in Table II illustrate the

TABLE I

LABILITY OF STOICHIOMETRIC PHOSPHORYLATION

Lysozyme disruption is described in METHODS, and mechanical disruption by agitation with glass beads in a Virtis homogenizer which has been described in a previous paper[4]. To break the cells by sonication, 50 g of fresh, packed cells were resuspended in 100 ml of cold 0.4 M sucrose–0.05 M NaCl and exposed to 10 1-min intervals of full intensity output with the Bronwill Biosonic II probe with 2-min cooling intervals. The same procedure was used for particle isolation in all experiments. To achieve mild osmotic shock, the particles, which are routinely resuspended in sucrose–NaCl medium at a concentration equivalent to 1 mg chlorophyll/ml, were diluted with 9 vol. cold distilled water and assayed immediately. For sonic treatment of the isolated particles, the preparation was diluted to 0.1 mg chlorophyll/ml with the sucrose–NaCl medium and exposed to maximum probe intensity for 2 min while surrounded with ice. Reaction mixtures for the measurement of $NADP^+$ reduction and phosphorylation contained the following components in μmoles in a total volume of 3 ml: $NADP^+$, 0.5 and saturating amounts of ferredoxin and NADP–ferredoxin oxidoreductase (EC 1.6.99.4) from *A. variabilis*; Tricine–NaOH (pH 7.8), 50; $MgCl_2$, 20; ADP, 10; sodium–potassium phosphate (pH 7.8), 3; and photosynthetic particles containing 30 μg chlorophyll. For the $Fe(CN)_6^{3-}$ reaction mixtures, the $NADP^+$, ferredoxin and NADP–ferredoxin oxidoreductase was replaced with 1 μmole $Fe(CN)_6^{3-}$; the Tricine (tris (hydroxymethyl) methyl glycine) buffer concentration was raised to 200 μmoles and the $MgCl_2$ was raised to 60 μmoles. Standard reaction conditions were: gas phase, air; illumination time, 5 min; light intensity, 14000 ft candles; temp., 25°. The reaction mixtures for the measurement of PMS cyclic phosphorylation contained the following components in μmoles in a total volume of 0.8 ml: PMS, 0.05; Tricine–NaOH (pH 6.8), 25; $MgCl_2$, 5; ADP, 5, sodium–potassium phosphate (pH 6.8), 3 and photosynthetic particles containing 30 μg chlorophyll. Standard reaction conditions for cyclic phosphorylation were: gas phase, N_2; illumination time, 10 min; light intensity, 5000 ft candles; temp., 25°.

Method of cell breakage	*$NADP^+$ reduction*	*Activity (μmoles/mg chlorophyll per h)*			*PMS phosphorylation*
		NADP phosphorylation	*$Fe(CN)_6^{3-}$ reduction*	*$Fe(CN)_6^{3-}$ phosphorylation*	
Lysozyme	86	107	257	171	376
Sonication	100	0	222	0	382
Mechanical disruption	90	0	278	0	280
Lysozyme					
Control	138	82	—	—	480
Particles diluted in H_2O	124	38	—	—	455
Control	126	116			348
Particles sonicated	80	23			220

basic observation. Particles prepared by sonication of the algae in 0.4 M sucrose–0.05 M NaCl were diluted 10-fold in 0.005 M Tris buffer (pH 7.8) to a chlorophyll concentration of 0.1 mg/ml and sonicated for 1 min at maximum intensity with a Bronwill Biosonic II probe. This lowers the cyclic phosphorylation activity. When the particles are separated from the soluble protein by centrifugation, then resuspended in 0.4 M sucrose–0.05 M NaCl, the PMS phosphorylation activity was greatly diminished or eliminated. Activity could be partially restored by adding the supernatant to the reaction mixture containing extracted particles. The restorative property of the supernatant fraction was heat labile and nondialyzable. The reactivating material in the supernatant fraction was purified by a procedure essentially identical to the one described by BIGGINS[1]. Fig. 1 describes the response of extracted particles to addition of the purified protein. Both PMS phosphorylation and the phosphorylation elicited by a combination of FMN and menadione responded similarly. The specific

TABLE II

REMOVAL AND RESTORATION OF A PROTEIN REQUIRED FOR CYCLIC PHOSPHORYLATION

The photosynthetic particles used in this experiment were isolated from sonically disrupted cells as described previously[4]. An aliquot of the preparation was diluted with 9 vol. of cold distilled water and sonicated for 5 min. The sonicated particles were then separated from the soluble protein by centrifugation at 104000 × *g* for 1 h.

	μmoles ATP/mg chlorophyll per h
Control	272
Sonicated	153
Sonicated, centrifuged-pellet	0
Sonicated, centrifuged-supernatant	0
Pellet + supernatant	168
Pellet + dialyzed supernatant	154
Pellet + boiled supernatant	0

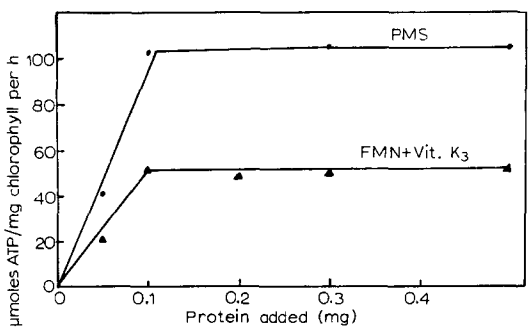

Fig. 1. The restoration of cyclic photophosphorylation activity with varying concentrations of purified phosphorylation protein. Reaction components and assay conditions are the same as those described in Table I, and the photosynthetic particles were prepared as described in Table II. In the reactions described by the lower curve PMS was replaced by 0.2 μmole FMN and 0.1 μmole vitamin K_3.

TABLE III

SPECIFICITY OF RESTORATION OF CYCLIC PHOSPHORYLATION

Reaction mixtures and assay conditions are the same as those described in Table I. Photosynthetic particles were depleted of essential protein as described in Table II. The amount of purified protein in mg added in these experiments was: cytochrome C_{554}, 0.016; ferredoxin, 0.133; plastocyanin, 0.045; phosphorylation protein, 0.065.

Additions	*μmoles ATP/mg chlorophyll per h*
None	14
Cytochrome C_{554}	0
Ferredoxin	28
Plastocyanin	20
Phosphorylation protein	150
All additions combined	104

activity of this protein in restoring phosphorylation is similar to that reported by BIGGINS[1]. The purified protein is still heterogeneous on disc gel electrophoresis, and its instability at this stage of isolation has hampered further purification. The purified protein fraction shows no obvious chromophore in the visible region of the spectrum, and it does not exhibit any overt ATPase activity.

Table III illustrates the specificity of this protein fraction in restoring photophosphorylation activity to depleted preparations. Cytochrome C_{554}, ferredoxin and plastocyanin prepared from this alga were inactive in restoring the cyclic phosphorylation. Furthermore these proteins give no increase in photophosphorylation when added in combination with the protein which does restore phosphorylation.

TABLE IV

SPECIFICITY IN RESTORATION OF ELECTRON TRANSPORT ACTIVITY

All reaction mixtures contained the following components in μmoles in a total volume of 3 ml: $NADP^+$, 1 and saturating amounts of ferredoxin and NADP–ferredoxin oxidoreductase; Tris–HCl (pH 7.8), 100; sodium–potassium phosphate (pH 7.8), 20; $MgCl_2$, 20; 2,3′,6-trichlorophenolindophenol, 0.15; sodium ascorbate, 10; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 0.3; and photosynthetic particles contain 30 μg chlorophyll. Standard reaction conditions were the same as in Table I. The amount of purified protein in mg added in these experiments was: plastocyanin, 0.04; cytochrome C_{554}, 0.024; phosphorylation protein, 0.1. Photosynthetic particles were depleted of essential protein by the procedure described in Table II.

Particles	*Addition*	*μmoles NADPH/mg chlorophyll per h*
Control	None	135
Extracted	None	66
	Plastocyanin	127
	Cytochrome C_{554}	98
	Phosphorylation protein	23
	All additions combined	102

Table IV shows that the extracted preparations suffer a loss of activity in the light-dependent transfer of electrons from reduced indophenol dye to $NADP^+$. This activity, as previously reported in detail[6], is restored by addition of plastocyanin, or to a lesser extent, by addition of cytochrome C_{554}. The purified protein fraction which restores cyclic phosphorylation activity has no restorative effect on this electron transport activity and is even inhibitory.

DISCUSSION

The number of ATP-synthesizing sites in the photosynthetic apparatus is an open question at this time. IZAWA AND GOOD[7] have reviewed the arguments for the existence of two phosphorylation reactions accompanying the flow of electrons from water to a terminal electron acceptor in noncyclic phosphorylation, and they have adduced new evidence for a stoichiometry of 2 ATP's per pair of electrons transported through the chain. The ratio of ATP synthesized to electron pairs transported to a terminal oxidant with blue-green algal preparations was greater than one in ten of the 26 preparations tested to date, suggesting the possiblity of two phosphorylation

sites in this chain. Our repeated failure to observe any stimulation of electron transport due to inclusion of phosphate or ADP in the reaction medium, or stimulation due to the addition of compounds which act as uncouplers with higher plant chloroplasts suggests that the algal stoichiometric phosphorylation is at best very loosely coupled to electron transport.

Several experiments point to a difference in inhibitor sensitivity between cyclic and stoichiometric phosphorylation in higher plant chloroplasts. AVRON AND SHAVIT[8] and DE KIEWIET *et al.*[9] have shown that stoichiometric phosphorylation is abolished at lower concentrations of carbonyl cyanide *p*-triflurometh oxyphenyl hydrazone than needed to abolish cyclic phosphorylation. The selectivity of desaspidin in eliminating cyclic but not stoichiometric phosphorylation is in dispute[10,11]. BLACK[12] has used hydrocarbons to inhibit completely stoichiometric phosphorylation while at the same time inhibiting cyclic phosphorylation by only 50 %. With the preparations from blue-green algae, stoichiometric phosphorylation is clearly more labile to physical abuse than either cyclic phosphorylation or the ability to transport electrons *via* the Hill reaction. These experiments imply the loss or dislocation of a coupling factor or phosphorylation component which is uniquely required for stoichiometric phosphorylation.

The cyclic phosphorylation activity of the blue-green algal preparations is considerably more stable than the stoichiometric phosphorylation, and it is only after sonication in dilute buffer that one can observe the reversible removal of protein required for this phosphorylation. The protein removed is not recognizable as an electron transport agent nor can it be replaced by several recognized redox carriers. Conversely, the protein required for photophosphorylation does not appear to be involved in the electron transport activity of the preparation. Since removal of the protein needed for cyclic phosphorylation involves simultaneous removal of much of the plastocyanin and some of the cytochrome C_{554}, it is important to note that restoration of phosphorylation is not enhanced by either plastocyanin or cytochrome C_{554}. However, the depleted particles require either plastocyanin or cytochrome C_{554} to transport electrons from reduced indophenol dye to $NADP^+$. This may mean that cyclic photophosphorylation involves a shorter or different electron transport chain than the reduced dye to $NADP^+$ reaction as suggested by ARNON *et al.*[13]. The site-specific inactivation of phosphorylation reported here is consistent with multiple paths of electron transport, but is not proof of completely independent systems.

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