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PHOTOPHOSPHORYLATION IN ISOLATED HETEROCYSTS FROM THE BLUE-GREEN ALGA *NOSTOC MUSCORUM*

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Isolated heterocysts of the blue-green alga *Nostoc muscorum (Anabaena* 7119) exhibit high rates of photophosphorylation in systems with cyclic and non-cyclic electron transport. Cyclic photophosphorylation mediated by *N*-methylphenazonium methosulfate is found to be sensitive to antimycin A, but not to 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). Non-cyclic electron transport (diaminodurol → methylviologen) coupled to phosphorylation is affected by DBMIB, but not by antimycin A. Studies with uncouplers indicate that ΔpH is the main component of the protonmotive force under continuous illumination. A different effect of NH_4Cl on dark- and photophosphorylation is observed and discussed with respect to localization of respiration in blue-green algae.

Introduction

Filamentous blue-green algae capable of nitrogen fixation under aerobic conditions have developed specialized cells called heterocysts [1]. These heterocysts provide the anaerobic environment necessary for operation of the oxygen-sensitive enzyme, nitrogenase. The O_2 concentration inside heterocysts is kept low by several mechanisms, including a modified photosynthetic apparatus which is not capable of O_2 evolution [2].

During differentiation of vegetative cells into heterocysts, Photosystem II becomes degraded. The remaining components of the photosynthetic electron transport chain are components of Photosystem I [3–5]. Linear photosynthetic electron transport with adequate electron donors and cyclic electron flow coupled to photophosphorylation are still possible. It has been postulated that an operating Photosystem I produces ATP and reducing equivalents to support the energy requirement of nitrogenase.

It has in fact been demonstrated that heterocysts are able to perform photosynthetic electron transport with artificial electron-donor and -acceptor systems [3,5]; the native electron donor, however, still remains open to speculation. There is only one report on photophosphorylation in isolated heterocysts showing low rates of ATP formation in the presence of PMS [3]. Apart from this report, no measurement of photophosphorylation in heterocysts has been published.

This is the first comprehensive study on photophosphorylation in isolated heterocysts, demonstrating high rates of photophosophorylation in systems with cyclic and non-cyclic (linear) electron transport. Furthermore, the effect of various electron-transport inhibitors and uncouplers has been investigated to gain further insight into the phosphorylating mechanisms in heterocysts. Comparative studies were made with spheroplasts prepared from the same alga.

Preliminary data showing photophosphorylation

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3(3,4-dichllorophenyl)-1,1-dimethylurea; PMS, *N*-methylphenazonium methosulfate; Tricine, *N*-tris-(hydroxymethyl)methylglycine; Chl *a*, chlorophyll *a*.

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with high rates in isolated heterocysts have been reported [6].

Materials and Methods

Algal culture: Nostoc muscorum (Anabaena 7119) was grown in axenic batch culture as described previously [5].

Isolation of heterocysts and preparation of spheroplasts. Algae were harvested by centrifugation in the early logarithmic phase of growth, washed once in a buffer comprising 0.5 M mannitol, 10 mM Na_2HPO_4/KH_2PO_4 (pH 6.7), 10 mM $MgCl_2$, 10 mM EDTA, and resuspended in this buffer. After addition of lysozyme (2 mg/ml), the algal filaments were incubated in the dark at 30°C with continuous stirring for 60 min. Subsequently, the algae were centrifuged and washed twice in a buffer containing 0.5 M mannitol, 10 mM Tricine/KOH (pH 7.8), 10 mM $MgCl_2$ and 5 mM Na_2HPO_4, to remove lysozyme. This procedure yielded spheroplasts, an aliquot of which was separated from the preparation and stored on ice until use for experiments.

For heterocyst isolation, spheroplasts were sonicated (3 × 5 s) with a Branson sonifier (type J 17 A), a procedure which destroyed vegetative cells, but left heterocysts intact. After several low-speed centrifugations (1 min at 1000 × *g*), heterocysts could be enriched from the sonicated algal suspension.

Measurement of photophosphorylation. Photophosphorylation was measured as incorporation of $^{32}P_i$ as described by Avron [7]. The reaction was performed in a Warburg apparatus in small glass vessels containing 10 mM Tricine/KOH (pH 7.8), 10 mM $MgCl_2$, 5 mM Na_2HPO_4/KH_2PO_4 (including $^{32}P_i$ with approx. 500000 cpm) and 2 mM ADP (total volume of 1 ml). In experiments with nigericin or valinomycin, the reaction medium additionally contained 10 mM KCl. For measurement of cyclic photophosphorylation, 50 μM PMS and 5 mM sodium ascorbate were added; for measurement of photophosphorylation coupled to a linear electron transport system, 0.1 mM diaminodurene, 5 mM sodium ascorbate, and 0.1 mM methylviologen were added. Reaction temperature was 25°C, light intensity 75 $W \cdot m^{-2}$ (white light). The reaction was started by addition of heterocysts or spheroplasts to the hypotonic reaction medium (final chlorophyll concentration, 10 μg/ml). After 3 min illumination, the reaction was stopped by turning off the light and adding 20 μl 60% $HClO_4$ to the reaction medium.

The samples were vigorously shaken and incubated on ice for 20 min to allow complete extraction. Subsequently, inorganic phosphate was removed from the reaction medium by extraction with isobutanol/benzene according to [7]. Radioactivity of esterified $^{32}P_i$ was measured in a liquid scintillation counter.

NADP photoreduction. Photoreduction of NADP by heterocysts and spheroplasts was measured with a Shimadzu UV 300 spectrophotometer equipped with a cross-illumination attachment type E. Measurements were made in the dual-wavelength mode with 340 nm as measuring beam and 400 nm as reference beam. Samples were illuminated by red light (Balzers filter K 65, light intensity 740 $W \cdot m^{-2}$ at the surface of the cuvette). The reaction medium (total volume 1 ml) contained 10 mM Tricine/KOH, (pH 7.8), 10 mM $MgCl_2$, 5 mM Na_2HPO_4, 2 mM ADP, 0.6 mM NADP and, if required, 0.1 mM diaminodurene, 5 mM sodium ascorbate, 0.05% Triton X-100. The chlorophyll concentration was 10 μg/ml. For quantitative evaluation, an extinction coefficient, ϵ_{340nm}, of 6.22 $mM^{-1} \cdot cm^{-1}$ for NADPH was used.

Measurement of oxygen uptake. Oxygen uptake was measured with a YSI oxygen monitor, model 53, at 25°C as described previously [5]. For further details see Table II.

Preparation of cytochrome c-553 and ferredoxin. Algal filaments were suspended in 50 mM Tris-HCl (pH 8.0) mixed with a double volume of glass beads (0.5 mm diameter) and broken in a Vibrogen Zellmühle as described elsewhere [8]. The resulting cell homogenate was centrifuged (60 min at 48000 × *g*) and the blue supernatant was subjected to ammonium sulfate fractionation. The 45–95% precipitate was dialyzed against 2 mM Tris-HCl (pH 8.0) and placed on top of a DEAE-52 cellulose column equilibrated with the same buffer. Ferredoxin and phycobiliproteins were adsorbed, whereas cytochrome *c*-553 passed through the column. It was concentrated by lyophilization or adsorption on a small CM-52-cellulose column equilibrated with 2 mM Tris-HCl (pH 8.0) and elution with a linear gradient (2 mM Tris-HCl (pH 8.0) containing 0–0.1 M NaCl). The cytochrome solution was then applied to a Sephacryl G-200 column (90 × 2.5 cm) equilibrated with 20 mM Tris-HCl (pH

8.0)/0.1 M NaCl. Cytochrome fractions with an absorbance ratio above 1.0 ($A_{317nm} : A_{276nm}$) were pooled and used for the experiments.

For purification of ferredoxin, the DEAE-52-cellulose column was washed with 20 mM Tris-HCl (pH 8.0)/0.2 M NaCl to remove phycobiliproteins. Subsequently, a linear gradient (20 mM Tris-HCl (pH 8.0) containing 0.2–0.4 M NaCl) was applied to elute ferredoxin. To remove nucleotides, the ferredoxin fractions were subjected to protamine sulfate precipitation. A neutralized 1% protamine sulfate solution was added to the ferredoxin solution until a slight turbidity was visible; after incubation on ice for 30 min, the solution was centrifuged and the precipitate discarded. The supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0)/0.35 M NaCl. After 2-fold dilution with water, the ferredoxin was again applied to a DEAE-52-cellulose column equilibrated with 20 mM Tris-HCl (pH 8.0)/0.18 M NaCl. Ferredoxin was eluted from this column with a linear gradient (20 mM Tris-HCl (pH 8.0) containing 0.2 M–0.5 M NaCl). The most purified fractions had an absorbance ratio of 0.4–0.5 ($A_{423nm} : A_{276nm}$).

Chemicals. Antimycin A, FCCP, and gramicidin D were obtained from Boehringer-Mannheim; CCCP was purchased from Sigma, Munich, and valinomycin from Serva, Heidelberg. Nigericin was kindly made available by Eli Lilly Laboratories, Indianapolis.

Results

Heterocysts exhibit significant rates of photophosphorylation in the absence of artificial electron-donor and -acceptor systems when ADP is added to the reaction medium. There is an endogenous rate (light minus dark) of 29 μmol ATP/mg Chl *a* per h. The rate of phosphorylation in the dark (22 μmol ATP/mg Chl *a* per h) can be stimulated by addition of ascorbate to 48 μmol ATP/mg Chl *a* per h.

Since Photosystem II is degraded in heterocysts, two systems related to Photosystem I activity have been chosen to investigate photophosphorylation further in isolated heterocysts, a system with cyclic electron flow in the presence of PMS/ascorbate and a system with linear (non-cyclic) electron flow with diaminodurene/ascorbate as electron-donor couple and methylviologen as electron acceptor. Addition of cytochrome *c*-553, which had been demonstrated to stimulate electron transport rates [6], had no effect on photophosphorylation in both systems tested (data not shown). NADP could not be taken as electron acceptor, because heterocysts prepared as described above photoreduce NADP only in the presence of added cofactors and Triton X-100. A requirement for Triton X-100 to demonstrate NADP photoreduction in heterocysts preparations has also been reported [9]. Addition of this detergent excludes any measurement of phosphorylation.

Table I summarizes the effects of various inhibitors on dark and light-induced phosphorylation in heterocysts and spheroplasts. There is no significant difference between heterocysts and spheroplasts concerning the action of inhibitors.

Action of uncouplers

CCCP and FCCP dissipate the proton gradient by a proton-shuttle mechanism and are, therefore, effective uncouplers (Fig. 1) acting on dark and photophosphorylation, with FCCP being more effective at equimolar concentrations. Gramicidin D, forming a proton channel through membranes leads to a similar decrease of phosphorylation rates. Nigericin (in the presence of K^+), mediating an electroneutral exchange of K^+ for H^+ strongly inhibits phosphorylation, whereas valinomycin (in the presence of K^+), performing an electrogenic transport of K^+ for H^+ has no significant effect on phosphorylation. Only in combination with NH_4Cl valinomycin causes a marked inhibition (cf. Ref. 10). NH_4Cl alone inhibits photophosphorylation up to 50% in both cyclic and non-cyclic electron transport systems, but leaves dark phosphorylation unaffected (Fig. 1).

Action of electron transport inhibitors

As expected, photophosphorylation in the cyclic and non-cyclic electron transport systems tested was not affected by DCMU (data not shown). This is in accordance with findings that heterocysts lack Photosystem II and associated components [3,5,11,12]. The plastoquinone antagonist DBMIB inhibits photophosphorylation as well as photosynthetic electron transport measured as oxygen uptake in the system diaminodurene/ascorbate → methylviologen (Table II). None of the other uncouplers tested had any effect on photosynthetic electron transport under these experimental conditions. Cyclic photophos-

TABLE I

EFFECT OF INHIBITORS ON PHOSPHORYLATION IN ISOLATED HETEROCYSTS AND SPHEROPLASTS

Control rates are taken as 100%; l, light; d, dark; l – d, light-minus dark; values represent averages of 3–5 experiments from different preparations.
A. Cyclic electron transport with PMS/ascorbate; control rates (μmol $^{32}P_i$ esterified/mg Chl *a* per h) heterocysts, l = 157 to 286, d = 36 to 58, l – d = 113 to 238 spheroplasts, l = 451 to 658, d = 41 to 62, l – d = 410 to 596.
B. Non-cyclic electron transport system (diaminodurene/ascorbate → methylviologen); control rates (μmol $^{32}P_i$ esterified/mg Chl *a* per h); heterocysts, l = 105 to 147, d = 30 to 48, l – d = 75 to 115; spheroplasts, l = 331 to 449, d = 41 to 53, l – d = 290 to 396.

	A. Cyclic						B. Non-cyclic					
	Heterocysts			Spheroplasts			Heterocysts			Spheroplasts		
	l	d	l – d	l	d	l – d	l	d	l – d	l	d	l – d
Control	100	100	100	100	100	100	100	100	100	100	100	100
CCCP ($5 \cdot 10^{-6}$ M)	56	59	52	65	54	68	57	53	58	70	61	54
FCCP ($5 \cdot 10^{-6}$ M)	35	52	31	42	48	40	35	53	30	38	43	31
Gramicidin D (1 μg/ml)	46	47	45	59	55	59	29	84	38	50	60	49
Nigericin ($1 \cdot 10^{-6}$ M)	34	66	18	27	39	23	35	49	26	31	45	27
Valinomycin ($1 \cdot 10^{-6}$ M)	93	100	92	102	98	103	85	76	87	91	77	92
NH_4Cl (5 mM)	62	78	54	65	92	53	55	100	42	54	98	51
Valinomycin ($1 \cdot 10^{-6}$ M) +NH_4Cl (5 mM)	11	21	14	5	29	2	7	34	5	3	41	0
Antimycin A ($1 \cdot 10^{-6}$ M)	54	69	60	55	70	62	90	110	89	97	94	103
DBMIB ($5 \cdot 10^{-6}$ M)	88	69	97	100	76	87	56	60	55	71	32	77

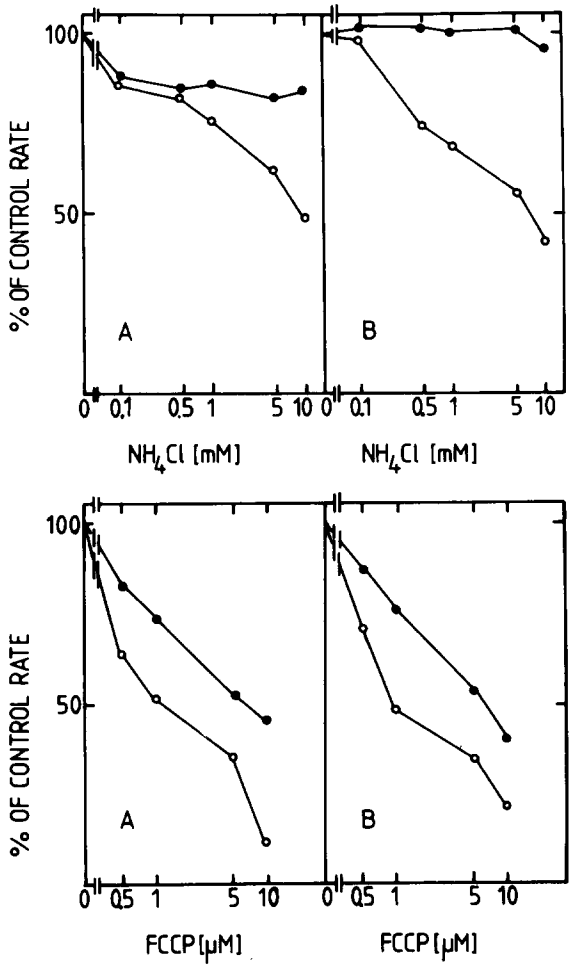

Fig. 1. Inhibition of dark- and light-induced phosphorylation of isolated heterocysts by FCCP and NH_4Cl. Control rates are taken as 100%; ○———○, rate in the light; ●———●, rate in the dark. (A) cyclic electron transport system with PMS/ascorbate; (B) non-cyclic electron transport system (diaminodurene/ascorbate → methylviologen). Each value represents the mean of three different experiments.

phorylation is DBMIB-insensitive, but inhibited by antimycin A. Photophosphorylation in the non-cyclic system is not affected by antimycin A (Fig. 2).

NADP photoreduction

Heterocysts perform NADP photoreduction in the presence of diaminodurene/ascorbate as electron donor, when cytochrome *c*-553, ferredoxin, an aliquot from the supernatant after sonication with the Branson sonifier, and Triton X-100 are added.

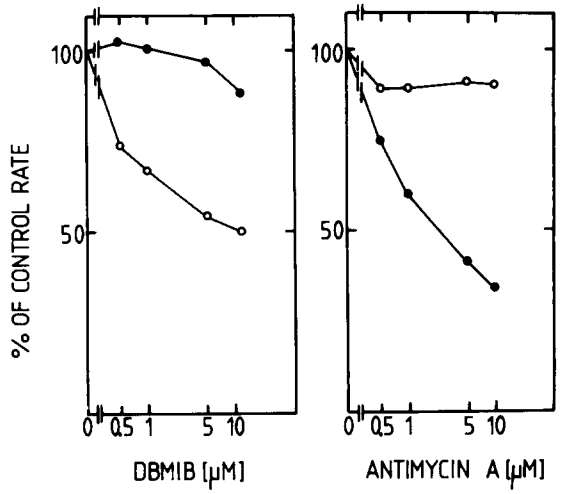

Fig. 2. Inhibition of cyclic and non-cyclic photophosphorylation in isolated heterocysts by DBMIB and antimycin A. Control rates (light minus dark) are taken as 100%; ○———○, photophosphorylation in a system with cyclic electron flow mediated by PMS/ascorbate; ●———●, photophosphorylation in a system with linear electron flow from diaminodurene/ascorbate → methylviolgen. Each value represents the mean of three different experiments.

Addition of the supernatant was without effect when heterocysts had been prepared by a more gentle procedure using a microsonic bath instead of the

TABLE II

INHIBITION OF PHOTOSYNTHETIC ELECTRON TRANSPORT (MEASURED AS O_2 UPTAKE) BY DBMIB IN ISOLATED HETEROCYSTS AND SPHEROPLASTS

The reaction medium contained 10 mM Tricine/KOH (pH 7.8), 10 mM $MgCl_2$, 5 mM Na_2HPO_4, 2 mM ADP, 0.1 mM methylviologen and 0.1 mM NaN_3. When required 0.1 mM diaminodurene, 5 mM sodium ascorbate, and $5 \cdot 10^{-6}$ M DBMIB were added. The chlorophyll concentration was 10 μg/ml. Final reaction volume: 1.5 ml. n.d., not determined. DAD, diaminodurene, MV, methylviologen.

Assay system	Inhibition (minus μmol O_2/mg Chl *a* per h)	
	Hetero-cysts	Spheroplasts
H_2O → MV	0	270
H_2O → MV + DBMIB	n.d.	60
DAD/ascorbate → MV	648	1576
DAD/ascorbate → MV + DBMIB	351	780

TABLE III

NADP PHOTOREDUCTION OF ISOLATED HETEROCYSTS AND SPHEROPLASTS

When required, 10 μM ferredoxin, 5 μM cytochrome *c*-553 and 0.05% Triton X-100 were added. cyt, cytochrome.

		μmol NADP reduced/mg Chl *a* per h
Spheroplasts	$H_2O \rightarrow$ NADP (+ferredoxin)	159
	DAD/ascorbate → NADP (+ferredoxin)	281
Heterocysts	DAD/ascorbate → NADP (+ferredoxin + cyt *c*-553 + Triton X-100	0
	DAD/ascorbate → NADP (+ferredoxin + cyt *c*-553 + Triton X-100 +supernatant	62
Heterocysts [a]	DAD/ascorbate → NADP (+ferredoxin + cyt *c*-553 + Triton X-100)	143

[a] Heterocysts were prepared by use of microsonic bath instead of the Branson sonifier.

Branson sonifier. These heterocysts could not be used for experiments of photophosphorylation because they were much less permeable to added substances. The component lost in our heterocyst preparations using the Branson sonifier is most likely to be ferredoxin-NADP-oxidoreductase which is solubilized by sonication. Rates of NADP photoreduction by heterocysts are somewhat lower than values obtained with spheroplast preparations. Spheroplasts perform NADP photoreduction which is only dependent on addition of ferredoxin (Table III).

Discussion

Our recent investigations [6] had demonstrated that isolated heterocysts exhibit cyclic and noncyclic photophosphorylation with high rates (light-minus-dark rate = 281 μmol ATP/mg Chl *a* per h) when adding adequate mediators, as well as appreciably high rates of endogenous photophosphorylation in the presence of ADP without added cofactors (light-minus-dark rate = 55 μmol ATP/mg Chl *a* per h). Our new preparations never reached these high values. In general, rates were reduced to about 50%. These decreased rates may be due to some unknown variation in the algal material. The rates measured for photophosphorylation with spheroplasts are comparable to those reported in the literature [13–16]. Concerning the action of various inhibitors tested, no basic difference between heterocysts and spheroplast preparations was observed, suggesting that the Photosystems I of both cell types operate similarly.

In the presence of uncouplers which affect ΔpH, strong inhibition of photophosphorylation is observed; Valinomycin (in the presence of K^+) which is thought to make only $\Delta\psi$ collapse, leaving ΔpH unaffected, has no effect on photophosphorylation. In contrast, nigericin (in the presence of K^+) causing ΔpH to collapse and leaving $\Delta\psi$ unaffected, strongly inhibits photophosphorylation, indicative of ΔpH being the main component of the protonmotive force ($\Delta\mu_{H^+}$) during continuous illumination. This has been documented for chloroplasts [17] and for spheroplasts and membrane preparations from blue-green algae [13,18].

NH_4Cl inhibits light-induced phosphorylation, but not dark phosphorylation, in heterocysts and spheroplasts. This is interesting with respect to localization of respiration in blue-green algae. If both respiratory and photosynthetic electron transport were located on the thylakoid membranes, it would be expected that NH_4Cl would have a similar effect on oxidative and photophosphorylation. If respiration were located on the cytoplasmic membrane and proton transport were directed from the cytoplasm to the outside of the cell, then a similar situation would exist as in mitochondria, where NH_4Cl is not an effective uncoupler [19]. In fact, it has been shown that upon energization of blue-green algae by an oxygen pulse or by light, protons are extruded into the medium [20]; on the other hand, thylakoids accumulate protons in their interior space upon illumination [13,14, 18]. From these data, Padan and Schuldiner [13] proposed a model suggesting an inverse direction of proton transport through thylakoid membranes and the cytoplasmic membrane in blue-green algae. Accord-

ingly, oxidative phosphorylation located on the cytoplasmic membrane should be less affected by NH_4Cl, which is indeed observed. Additional evidence was reported by Scherer et al. [21] who demonstrated that respiration and photosynthesis show different break temperatures in the Arrhenius plots, excluding a common electron transport chain.

Inhibition of photophosphorylation by DBMIB in the non-cyclic electron transport system with diaminodurene/ascorbate as electron donor is due to inhibition of electron transport. This suggests that at least part of the electrons from diaminodurene are fed into the electron transport chain via the FeS-Rieske center, the reduction of which is blocked by DBMIB [22]. Oxidation of reduced diaminodurene may be proceed analogous to oxidation of plastoquinone.

Antimycin A has been shown to inhibit ferredoxin-catalyzed cyclic electron transport in chloroplasts, but not cyclic electron transport with artificial mediators like PMS [23,24]. In contrast to these reports, we have found that cyclic photophosphorylation with PMS in heterocysts and spheroplasts is sensitive to antimycin A. The cyclic PMS system seems to involve cytochrome *b*-563, the redox reactions of which are thought to be affected by antimycin A. However, it should be mentioned that the effects of antimycin A on light-induced electron transport in chloroplasts are rather obscure (cf. Ref. 25). Its mode of action had been mainly deduced from investigations with mitochondria [26]. It may be concluded from the experiments with DBMIB and antimycin A that different parts of the native electron transport chain are involved in non-cyclic and cyclic photophosphorylation.

Experiments with chloroplasts from *Bumilleriopsis* revealed that soluble cytochrome *c*-553 is necessary for effective photophosphorylation with PMS [27]. As our heterocyst preparations lose substantial amounts of soluble cytochrome *c*-533 after sonication, rates of photophosphorylation in the cyclic and non-cyclic electron transport system (both of which require cytochrome *c*-533) are suboptimal.

Even our previously reported high rates of photophosphorylation in isolated heterocysts [6] are not sufficient to support nitrogenase activity completely. The question of whether heterocysts are able to provide enough ATP via cyclic photophosphorylation to account for the energy requirement of nitrogenase remains to be answered. Recent investigations [28] have indicated that the membrane potential might be an important regulator of nitrogenase activity.

Acknowledgments

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