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Respiration in Blue-Green Algae

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The low rate of endogenous respiration exhibited by the blue-green algae *Anacystis nidulans* and *Phormidium luridum* was not increased by the addition of respiratory substrates. However, endogenous respiration was inhibited by low concentrations of cyanide and by high carbon monoxide tensions. In addition, the uncouplers dinitrophenol and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone both stimulated the respiratory rate. The transition of cells from the aerobic steady state to anaerobiosis was accompanied by a decrease in the concentration of cellular nicotinamide adenine dinucleotide phosphate ($NADP^+$) and adenosine triphosphate (ATP), whereas the concentration of nicotinamide adenine dinucleotide (NAD^+) was unchanged. Concomitant with the metabolite decreases were stoichiometric increases io reduced $NADP^+$ (NADPH), adenosine diphosphate, and adenosine monophosphate. A decrease in ATP was also observed after the addition of uncouplers. These data are interpreted as evidence for the association of oxidative phosphorylation with the oxidation of $NADP^+$-linked substrates in these algae. Membrane fragments isolated from the algal cells oxidized succinate, malate, ferrocytochrome *c*, ascorbate-tetramethyl-*p*-phenylenediamine, and reduced 2,6-dichlorophenol indophenol but did not oxidize NADPH or reduced NAD^+ in a cyanide-sensitive system. Oxidative phosphorylation has not yet been demonstrated in these fragments, but a dark ATP-P_i exchange, distinct from the light-triggered exchange associated with photosynthesis, is readily observed. This exchange was inhibited by phloridzin, Atabrine, and uncouplers in concentrations which suggest that the mechanism of oxidative phosphorylation in blue-green algae is different from that found in other bacteria and in mitochondria. These results led to the conclusion that the biochemical basis for obligate autotrophy in these organisms does not lie in the metabolic events associated with terminal electron transport and energy conservation.

The blue-green algae are procaryotic organisms whose relationship to the bacteria has recently received renewed interest (9, 11, 15). Although the blue-green algae are morphologically simple, their level of metabolic complexity is quite high. For example, their photosynthetic mechanism is similar to that observed in the higher plants: two photoacts are necessary and the high potential oxygen evolution mechanism is present (3, 8).

The respiratory mechanism of these algae is unknown but there are several reports describing an unusual effect of light on this process in vivo (5, 10, 13). At low intensities, light suppresses oxygen uptake, whereas at high intensities it is stimulatory. The effect is sensitized by chlorophyll and the stimulation is inhibited by the photosystem II inhibitor, 3,4-dichlorophenyldimethylurea (10). Thus, there appears to be a rather intimate relationship between respiration and photosynthesis in these algae, and, in view of the absence of any obvious respiratory assemblies that can be deduced from ultrastructural studies (15, 22), the possibility exists that the processes of photosynthesis and respiration may share certain components. Thus, the two processes of energy conversion may be functionally related as is the case for the photosynthetic bacteria (20).

Webster and Frenkel (21) showed that the endogenous respiration of *Anabaena* is inhibited by cyanide, azide, and CO. They suggested that the terminal oxidase is a hemoprotein. Horton (12) reported that particles isolated from *Anacystis nidulans* and *Anabaena variabilis* possess a cyanide-sensitive reduced nicotinamide adenine dinucleotide (NADH):O_2 oxidoreductase but do not possess cytochrome *c*:O_2 oxidoreductase, succinate:O_2 oxidoreductase, or NADH:cytochrome *c* oxidoreductase.

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Smith, London, and Stanier (19) reported the absence of NADH:O_2 oxidoreductase and α-ketoglutarate dehydrogenase in preparations from *A. nidulans*, *Coccochloris peniocystis*, and *Gloeocapsa alpicola*. They suggested that these deficiencies represent the biochemical basis for obligate autotrophy in these organisms. In addition, they speculated that the process of oxidative phosphorylation may not exist and that all adenosine triphosphate (ATP) is generated by substrate-level phosphorylations accompanying glycolysis.

This paper reports that the electron transport in *A. nidulans* and *Phormidium luridum* arises from nicotinamide adenine dinucleotide phosphate ($NADP^+$)-linked substrates and that oxidative phosphorylation accompanies the electron flow to a hemoprotein terminal oxidase.

MATERIALS AND METHODS

Organisms. *A. nidulans* and *P. luridum* were obtained from the Culture Collection, Indiana University, Bloomington, and were cultured photoautotrophically as described previously (2, 14).

Cell breakage and fractionation. Cells were harvested and washed in 0.5 M sucrose, 50 mM *N*-tris-(hydroxymethyl)methyl-2-amino ethane sulfonic acid (TES) or *N*-2-hydroxyethylpiperazine *N*-2-ethane sulfonic acid (HEPES; *p*H 7.4), and 10 mM $MgCl_2$ and were finally suspended in this buffer to give about a 25% (w/v) cell suspension. After cell breakage by ultrasound (10 kc, 1 min) or high pressure extrusion (15,000 psi), the homogenate was centrifuged at 3,000 $\times$ *g* for 10 min to remove whole cells and large fragments. The supernatant fluid was centrifuged at 48,000 $\times$ *g* for 30 min to sediment a membrane fraction which was used for experiments involving substrate oxidation.

Protoplasts from *P. luridum* were prepared by using muramidase (EC 3.2.1.17) as described previously (2). These protoplasts were used in the ATP-P_i exchange and oxidative phosphorylation experiments.

Biochemicals. D-Glucose-6-phosphate:$NADP^+$-oxidoreductase (EC 1.1.1.49), alcohol:nicotinamide adenine dinucleotide (NAD^+) oxidoreductase (EC 1.1.1.1), ATP:D-hexose-6-phosphotransferase (EC 2.7.1.1), L-lactate:NAD^+ oxidoreductase (EC 1.1.1.27), and ATP:adenosine monphosphate (AMP) phosphotransferase (EC 2.7.4.3) were obtained from Boehringer Manheim Corp. Cytochrome *c*, adenine nucleotide phosphates, and nicotinamide adenine dinucleotides were also obtained from Boehringer Manheim Corp. The buffers TES and HEPES were obtained from Calbiochem, Los Angeles, Calif.; antimycin A, 2*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), oligomycin, phloridzin, and Atabrine were obtained from Sigma Chemical Co., St. Louis, Mo.

General assays. All reactions were performed at 25 C. Oxygen uptake was measured either manometrically or polarographically by using a covered Clarke oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The spectrophotometric and polarographic assays for the oxidation of NADH, reduced NADP (NADPH), succinate, malate, reduced 2,6-dichlorophenol indophenol ($DPIPH_2$), and ferrocytochrome *c* by particle preparations from the algae were performed as described previously (3).

The ATP-P_i exchange was measured in the dark by using radioactive orthophosphate in a reaction mixture (1.5 ml) containing 50 mM TES or HEPES buffer (*p*H 7.4), 3.3 mM ATP, 3.3 mM $K_2H^{32}PO_4$, 10 mM $MgCl_2$, and algal protoplasts equivalent to 30 to 70 μg of chlorophyll a. Radioactive ATP was recovered from the deproteinized reaction mixtures by the isobutanol-benzene phase separation method described by Avron (1). Samples (100 μliters) were absorbed on filter paper and immersed in a solution containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene; their radioactivity was determined directly by using a Packard automatic scintillation spectrometer (model 3320)

For metabolic steady-state experiments, the algae were suspended in growth medium containing 30 μliters of silicone antifoam emulsion (SAG 5441) per liter, a gift from Union Carbide, New York, N.Y., at densities which varied from 10 to 30 mg (dry weight) per ml. The cells were equilibrated in a 50-ml chromatographic column fitted with a medium sintered-glass filter and rapidly sparged with air. At intervals, 2-ml samples were withdrawn for metabolite analyses. For the assay of $NADP^+$, NAD^+, ATP, adenosine diphosphate (ADP), and AMP, the cells were extracted with 3.5% perchloric acid. For the assay of NADPH and NADH, the cells were extracted with 30% ethyl alcohol, 0.3 N KOH. To ensure that adequate cell breakage had occurred, the samples were subjected to freezing and thawing three times. After removal of insoluble material by centrifuging the samples at 5,000 $\times$ *g* for 10 min, the cell extracts were neutralized. Assays for certain cellular metabolites were conducted immediately.

Experiments designed to measure the concentration of metabolites during the transition of cells to anaerobiosis were performed in a specially constructed chamber which allowed the continuous monitoring of oxygen tension polarographically. A hydraulically assisted sampling device permitted withdrawal of samples from the chamber at any desired oxygen concentration. Anaerobiosis was induced by depleting the oxygen in the medium by cell respiration during the course of the experiment.

Adenine nucleotide phosphates were measured spectrophotometrically by slight modification of the specific enzyme assays described by Williamson and Herczeg (24). However, the measurement of $NADP^+$(H) and NAD^+ (H) by such enzymatic analyses and fluorometric or spectrophotometric detection was found to be impossible because of the intense color and background fluorescence of the cellular extracts. These metabolites were successfully determined in a cyclic system in which NADPH or NADH was generated specifically by either D-glucose-6-phosphate:$NADP^+$-oxidoreductase or alcohol:NAD^+ oxidoreductase, respectively, and these reduced forms were coupled to 2,6-dichlorophenol indophenol (DPIP) via phenazine methosulfate (PMS; 18). The

rate of DPIP reduction in a reaction limited by the concentration of nicotinamide adenine dinucleotide was measured spectrophotometrically at 600 nm. This rate was found to be proportional to the concentration of NAD^+ (H) or $NADP^+$ (H) in the region of 0.5 to 5.0 μM. The following reaction mixtures (2 ml) were used: for determination of NAD^+ and NADH, 50 mM triethanolamine, 10 mM $MgCl_2$, 5 mM ethylenediaminetetraacetic acid (EDTA) buffer (*p*H 7.4), 86 mM ethyl alcohol, 100 μM PMS, 50 μM DPIP, and 6.0 IU of alcohol:NAD oxidoreductase; for determination of $NADP^+$ and NADPH, 50 mM triethanolamine, 10 mM $MgCl_2$, 5 mM EDTA (*p*H 7.4), 0.5 mM glucose-6-phosphate, 100 μM PMS, 50 μM DPIP, and 0.3 IU of glucose-6-phosphate:$NADP^+$ oxidoreductase.

Chlorophyll a was determined spectrophotometrically using the molar extinction coefficient, $\epsilon^{1\,em}_{680nm} = 82{,}000$.

RESULTS

Endogenous respiration in vivo. The dark endogenous rate of respiration of *A. nidulans* and *P. luridum* was found to be between 4 and 6 μliters of O_2 per mg (dry weight) per hr, and it remained constant for about 8 hr after harvesting the cells from a photoautotrophic culture. The respiratory rate was not improved by the addition of respiratory substrates such as sugars or organic acids. This confirms the results of Kratz and Myers (14). An upper limit for the K_m of the oxidase could be estimated from the polarographic data. For both organisms the K_m was about 5 μM, indicating that the terminal oxidase has a very high affinity for oxygen. The respiratory rate was inhibited 43% by a 19:1 CO-O_2 mixture, and complications resulting from photosynthetic oxygen evolution did not permit us to test the photoreversibility of this inhibition. The oxidase system was quite sensitive to cyanide (Fig. 1). These results support the earlier suggestion of Webster and Frenkel (21) that the terminal oxidase in these organisms is probably a hemoprotein.

Although respiration in *P. luridum* was completely inhibited by cyanide, approximately 20% of normal respiration remained in *A. nidulans* in 0.4 mM CN^-, suggesting the presence of alternate pathways to oxygen in this organism.

Stimulation of the respiratory rate by low concentrations of the uncouplers dinitrophenol (DNP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) suggests that oxidative phosphorylation is associated with electron transport in these organisms.

Antimycin (0.3 mM), HOQNO (0.7 mM), and oligomycin (10 μg/ml) had no effect on the respiratory rate.

Metabolic steady states. The intracellular pool size of various metabolites associated with respiration were measured in a series of experiments in which the aerobic steady state of the cells was

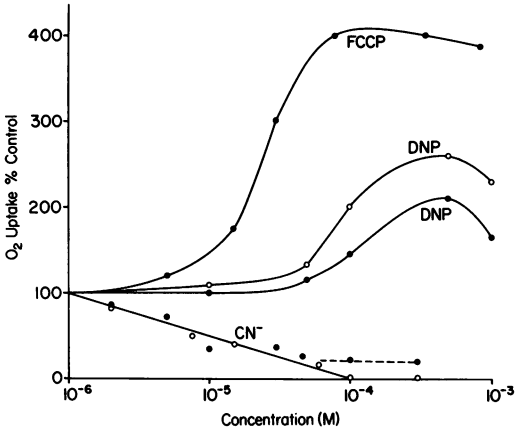

FIG. 1. *Sensitivity of endogenous dark respiration by A. nidulans (●) and P. luridum (○) to cyanide and uncouplers. The control rates of respiration were between 4.3 and 5.9 μliters of O_2 per mg (dry weight) per hr.*

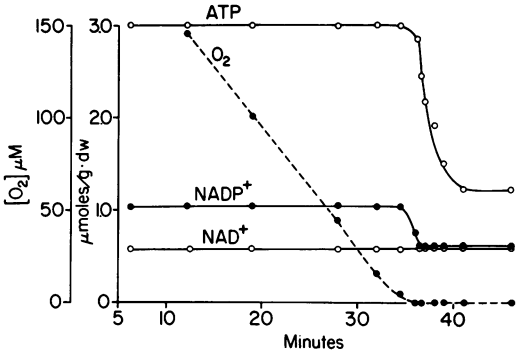

FIG. 2. *Changes in the cellular pool sizes of metabolites in A. nidulans, accompanying a transition from the aerobic steady state to anaerobiosis. Oxygen depletion was by cellular respiration in a sealed chamber. The concentration of oxygen at zero time was 240 μM.*

perturbed by some agent. Figure 2 illustrates some changes induced in *A. nidulans* during a transition of the cells to anaerobiosis. No changes in the concentration of ATP, NAD^+, and $NADP^+$ occurred until the oxygen tension was lowered to 5 μM. Concentrations lower than this resulted in a reduction in the concentration of ATP and $NADP^+$. Table 1 summarizes data from a series of similar experiments. In all cases, the perturbation was monitored by sampling at 20-sec intervals through the transition period. Nitrogen, cyanide, and anaerobiosis (via cellular respiration) all produced a reduction in the cellular concentration of ATP and $NADP^+$, whereas NAD^+ was unchanged. The decrease of ATP was accompanied by an increase of ADP and AMP, and, similarly,

TABLE 1. *Equilibrium metabolite pool sizes in A. nidulans for a variety of conditions and in the presence of various agents*

Metabolite	Pool size (μmoles/g, dry wt, of cells)					
	Aerobic	Anaerobic	N_2	2 mM CN	55 μM FCCP	100 μM DNP
ATP	3.02	1.20	1.50	1.35	0.60	1.06
ADP	1.26	2.50			2.98	1.43
AMP	0.18	0.62			0.91	
NAD^+	0.59	0.59	0.60	0.58		
NADH	0.33	0.33		0.32		
$NADP^+$	1.04	0.60	0.51	0.47		
NADPH	0.54	1.01		1.10		

the decrease in $NADP^+$ resulted in a stoichiometric increase in the reduced species. The uncouplers FCCP and DNP both induced an even greater reduction in the concentration of ATP, but the concentration never reached zero. For FCCP, the effect was half maximal at 10 μM. This suggests that in the uncoupled condition a small pool of ATP is maintained by substrate-level phosphorylation. Similar data were obtained for *P. luridum*, but sampling was sometimes erratic because the organism is filamentous.

These data show that the stimulation of oxygen uptake in vivo by uncouplers is accompanied by a reduction in the concentration of cellular ATP. This is interpreted as unequivocal evidence for oxidative phosphorylation in these organisms. Of the two species of nicotinamide adenine dinucleotides, only the $NADP^+$ to NADPH ratio was affected by agents which block electron flow. Hence, it is suggested that the oxidation of endogenous substrates by these organisms is mediated by some $NADP^+$-linked enzymes rather than by NAD^+-linked enzymes.

Oxidative activities at the subcellular level. Oxidative activities were investigated in membrane preparations isolated from algae which had been disrupted by ultrasound, high-pressure extrusion, sand-grinding, or protoplast lysis. NADPH-oxygen oxidoreductase was expected in view of the in vivo data described above. However, the only oxidation of NADPH that could be measured was cyanide-insensitive and required the soluble fraction of the cell in addition to the membrane preparation (Table 2). It thus appears that the cyanide-sensitive NADPH:O_2 oxidoreductase predicted from the experiments with whole cells may have been destroyed during cell breakage. The observed cyanide-insensitive oxidation of NADPH can be accounted for by assigning this activity to ferredoxin, a soluble, nonheme iron protein, and $NADP^+$:ferredoxin oxidoreductase (EC 1.6.99.4), a flavoprotein which is normally tenaciously bound to photosynthetic lamellae. This enzyme system mediates photosynthetic $NADP^+$ reduction and has been observed to act reversibly in chloroplasts by oxidizing NADPH in the dark after a period of photoreduction (16). Furthermore, it is possible that the $NADP^+$:ferredoxin oxidoreductase could also be a participant in the observed diaphorase activity between NADPH, NADH, and DPIP. NADH:oxygen oxidoreductase (NADH oxidase) activity was not demonstrated or expected from the in vivo studies, and I thus confirm the results of Smith et al. (19) but disagree with those of Horton (12).

Low activities of succinic and malic dehydrogenase were stimulated by cyanide (10 μM), and, in common with many other bacterial oxidase systems, the malic dehydrogenase was found to be independent of exogenous NAD^+.

Succinate, ascorbate-tetramethyl-*p*-phenylene diamine (TMPD), cytochrome *c*, and $DPIPH_2$ were oxidized by the particles in a pathway which is inhibited by cyanide. The cyanide-inhibition profile was similar to that for endogenous respiration of the cells, indicating the participation of the same terminal oxidase. These experiments also showed that the terminal oxidase is a component of the membrane.

The membranes were incapable of directly oxidizing sugars such as glucose, fructose, mannose, and glucose-6-phosphate.

Phosphorylation mechanism. All efforts directed at a demonstration of oxidative phosphorylation coupled to the oxidation of succinate, malate, NADPH, ascorbate-TMPD, and ferrocytochrome *c* were negative, even when using membrane preparations and lysed protoplasts which were very active in photophosphorylation (3). However, partial reactions of oxidative phosphorylation, such as adenosine triphosphatas, and ATP-

TABLE 2. *Oxidative activities by particle preparations from P. luridium*

System		Activity[a]	Concn of CN^- required for 50% inhibition (μM)
Electron donor	Electron acceptor		
Cytochrome *c*[b]	O_2	83	3.1
$DPIPH_2$[b]	O_2	73	7.9
Succinate	O_2[b]	42	2.7
NADH	DPIP[b]	1,520	NE[c]
NADPH	DPIP[b]	2,080	NE
NADH[b]	O_2	0	NE
NADPH[b]	O_2	15	NE
Ascorbate-TMPD	O_2[b]	31	
Succinate	PMS, DPIP[b]	61	—[d]
Malate	PMS, DPIP[b]	43	—[e]

[a] The protein to chlorophyll ratio of the particles was found to be approximately 6.9. Values expressed as nanomoles per minute per milligram of chlorophyll a.
[b] Indicates component measured in assay and referred to in the activity column.
[c] No effect at millimolar concentration.
[d] Stimulated 15% by 10 μM.
[e] Stimulated 11% by 10 μM.

P_i exchange, were readily demonstrable. The ATP-P_i exchange activity appeared to be different from the light-triggered exchange associated with the photosynthetic apparatus (6). It occurred in the dark and did not require the presence of any redox cofactors such as PMS or sulfhydryl agents. Table 3 shows the sensitivity of the exchange to various high-energy intermediate inhibitors and uncouplers. The low concentrations of FCCP and the abnormally high concentrations of oligomycin required for inhibition suggests that the oxidative phosphorylation mechanism in *P. luridum* is quite different from that found in other bacteria and mitochondria.

TABLE 3. *Sensitivity of ATP-P_i exchange by lysed protoplasts and particle preparations from P. luridum*

Agent	Concn required for 50% inhibition[a] mM
Phloridzin	500
Atabrine	17
Gramicidin	—[b]
Oligomycin	2
DNP	9
FCCP	0.1
NH_4^+	—[c]
ADP	—[d]

[a] The control rates for the ATP-P_i exchange varied between 6.9 and 14.6 μmoles exchanged per hr per mg of chlorophyll a, with an average of 8.5.
[b] No effect at 7 μM/ml.
[c] No effect at 10 mM.
[d] Equimolar with ATP.

DISCUSSION

A. nidulans and *P. luridum* both have a low rate of endogenous respiration which is strongly inhibited by cyanide and CO. Therefore, it is suggested that the functional oxidase is a hemoprotein rather than a flavoprotein. My experiments on the oxidation of various substrates in vitro also show that the oxidase is a component of the membrane fraction. Investigations on the perturbation of the steady-state pool sizes of respiratory metabolites by a variety of agents suggest that oxidative phosphorylation is associated with the oxidation of endogenous substrates in these organisms and that some of the oxidative enzymes are linked to $NADP^+$. The appropriate conditions and substrates have not yet been found for the demonstration of oxidative phosphorylation in vitro. However, my studies on the related, dark ATP-P_i exchange associates this activity with the membrane fraction, which also mediates electron flow and contains the terminal oxidase. The precise intracellular location of these activities will be resolved by current studies on the density gradient fractionation of the plasma membrane and the cytoplasmic lamellar system, which is the site of the photosynthetic apparatus (17).

This study confirms the data of Smith et al. (19) with respect to the lack of NADH oxidase in these algae, and it is suggested that the discrepancy with the data of Horton (12) for photoheterotrophically grown *A. nidulans* can be attributed to the growth conditions of the organisms. For

photoautotrophically grown cells, my data predict the existence of an NADPH-oxidase rather than an NADH-oxidase. This is consistent with the published reports concerning carbohydrate dissimilation in blue-green algae; these reports show that the oxidative pentose cycle is the major pathway (7, 23).

In their general hypothesis for obligate autotrophy, Smith et al. (19) suggested that the absence of an NADH-oxidase would also result in a lack of oxidative phosphorylation and would prove to be a basic obstacle for heterotrophic growth. They also suggested that the only conceivable mechanism for ATP generation is substrate level phosphorylation accompanying glycolysis. This general hypothesis appears to require substantial revision in the case of the two blue-green algae *A. nidulans* and *P. luridum*. Therefore, the basis for obligate autotrophy must reside in some aspect of metabolism other than the terminal electron transport pathway and the associated energy-conservation mechanism.

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ADDENDUM IN PROOF

Since the submission of this article to press, Leach and Carr (Biochem. J. 112:125–126) have demonstrated the coupling of oxidative phosphorylation to NADPH oxidation by a cell-free preparation from *Anabaena variablis*. Also, Bisalputra, Brown, and Weier (J. Ultrastruct. Res. 27:182–197) have shown the deposition of reduced tellurite and tetrazolium on the internal lamellae but not on the plasma membrane of *Nostoc sphaericum*, indicating that the photosynthetic membranes are also functional in respiration.

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