PHOTOLYSIS OF WATER COUPLED TO NITRATE REDUCTION BY *Nostoc muscorum* SUBCELLULAR PARTICLES

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SUMMARY. Subcellular particles from the blue-green alga *Nostoc muscorum* contain ferredoxin-nitrate reductase and can use NADPH to reduce nitrate in a ferredoxin-dependent reaction through a NADP-reductase system. Likewise, evidence is provided that a ferredoxin-dependent photoreduction of nitrate to nitrite by water coupled to stoichiometric oxygen evolution takes place in cell-free preparations of this alga.

The nitrate-reducing system of several blue-green algae has been recently characterized (1-5). In *Anabaena cylindrica*, as well as in *Anacystis nidulans*, nitrate reductase activity has been found either tightly bound to photosynthetic particles or in soluble form, depending on the technique used to disrupt the algal cells (1,2). The enzyme is ferredoxin-dependent (1-3), a property shared with all the photosynthetic procaryotes so far studied (6).

Nitrate reduction has been achieved by using several ancillary systems as electron donors, *e.g.* chemically reduced viologen dyes, ferredoxin-NADP reductase from spinach and the couple ascorbate/ DPIP together with the photosystem I (1,2).

In this paper, nitrate reduction with subcellular particles of

Abbreviations: DPIP: 2,6-dichlorophenol-indophenol; CMU: 3-(4-chlorophenyl)-1,1-dimethylurea.

the blue-green alga *Nostoc muscorum* by NADPH through the ferredoxin-NADP reductase system is presented. Moreover, evidence is shown that ferredoxin-dependent nitrate photoreduction by these subcellular particles can use the reducing power coming from water. Stoichiometric oxygen evolution coupled to photoreduction of nitrate to nitrite by water is also reported.

MATERIALS AND METHODS

N. muscorum cells (strain 7119 from the Department of Plant Physiology, Berkeley, USA, kindly supplied by Dr. D.I. Arnon) were cultivated autotrophically in the light (5,000 lux) at 25°C on 5% CO_2 in air, using the culture medium described by Arnon *et al.* (7) but with KNO_3 (20 mM) as nitrogen source. The algal cells were harvested in the mid-logarithmic phase of growth by centrifuging at 3,000 x g for 5 min, and frozen after washing with 50 mM Tris-HCl buffer, pH 7.5. The frozen cells were subsequently thawed in 10 mM Tris-HCl, pH 7.5, and disrupted by magnetic stirring at room temperature for 2 h, in the presence of lysozyme (4 mg/g fresh weight). The homogenate was then spun down at 3,000 x g for 5 min and the resulting supernatant was centrifuged at 40,000 x g for 15 min. The pellet was finally resuspended in 50 mM Tris-HCl buffer, pH 7.5 and used as particulate system A.

Another method was also applied to obtain photosynthetic particles capable of reducing nitrate. 3-days old algal cells were harvested at low speed centrifugation (3,000 x g for 10 min) and washed with 50 mM Tricine-KOH buffer, pH 7.7, containing 10 mM $MgCl_2$ and 0.5 mM sucrose. The washed cells were resuspended in the same buffer and then disrupted in the cold with a Sonifier Branson 12 (20 Kc/70 W) for 40 sec. About 1 mg each of DNAase and RNAase was then added per about 40 ml of slurry. The broken material was centrifuged at 3,000 x g for 5 min and the supernatant was spun down at 40,000 x g for 20 min. The pellet was washed with the foregoing Tricine-KOH buffer and centrifuged again in the same conditions. The sediment resuspended in Tricine-KOH buffer was used as particulate system B. The pellet obtained after the first centrifugation of the sonicated cells was subjected to a second sonication and treated in the same way as described for the preparation of B particles. The resulting particulate system (C) was used to study the oxygen evolution coupled to nitrate reduction by water.

Nitrate reductase activity was followed by appearance of nitrite (8). Special experimental conditions for activity assays are described in the captions of the corresponding Tables. *N. muscorum* ferredoxin was prepared as indicated by Mitsui & Arnon (9). Spinach NADP-reductase was purified according to Shin *et al.* (10). Chlorophyll was determined as described by Arnon (12). Protein was estimated by the Folin-phenol reagent (11).

Oxygen evolution was measured with a Clark-type electrode.

TABLE I

FERREDOXIN-DEPENDENT NITRATE REDUCTION WITH NADPH BY *Nostoc muscorum* PARTICLES

System	Nitrite formed (nmoles)
Complete	148
Minus NADPH	9
Minus ferredoxin	10
Minus nitrate	0
Minus particles	1

The complete system included in a final volume of 1 ml: 100 µmoles Tris-HCl buffer, pH 8.5; 40 µmoles KNO_3; 0.2 mg *N. muscorum* ferredoxin; 2.4 µmoles NADPH; *N. muscorum* particles A containing 10 mg protein. The assay was carried out under air at 30°C for 30 min

RESULTS AND DISCUSSION

As it has been reported for other blue-green algae (1,2), *Nostoc muscorum* nitrate reductase remained associated with particles. These subcellular particles can use the following electron donors for nitrate reduction: methyl viologen chemically reduced with dithionite, and ascorbate/DPIP through photosystem I, in a ferredoxin-dependent light reaction. Moreover, NADPH was an effective electron donor in the ferredoxin-dependent reduction of nitrate to nitrite catalyzed by *N. muscorum* particles (Table I). In contrast, NADH could not function even in presence of added FAD, which slightly stimulated the NADPH-ferredoxin-de-

TABLE II

EFFECT OF REDUCED PYRIDINE NUCLEOTIDES AND FAD ON THE FERREDOXIN-DEPENDENT NITRATE REDUCTASE ACTIVITY OF *Nostoc muscorum* PARTICLES

System	Nitrite formed (nmoles)
NADH	0
NADH, ferredoxin	0
NADH, FAD	0
NADH, ferredoxin, FAD	0
NADPH	1
NADPH, ferredoxin	37
NADPH, FAD	1
NADPH, ferredoxin, FAD	58

The reaction mixture contained in a final volume of 1 ml: 100 µmoles Tris-HCl, pH 8.5; 40 µmoles KNO_3, and where indicated 0.2 mg *N. muscorum* ferredoxin; 2.4 µmoles NADPH; 2.4 µmoles NADH; 20 µmoles FAD; *N. muscorum* particles A containing 4.5 mg protein. The reaction was carried out under air at 30°C for 30 min.

pendent nitrate reductase activity (Table II). When FAD was substituted for FMN, no stimulation of activity was observed results not shown). It has been reported that in *A. cylindrica* (2), NADH behaved effectively as electron donor in the reduction of nitrate by subcellular particles and no increase in activity was observed upon addition of either ferredoxin or FAD or FMN; NADPH, however, required ferredoxin when used as reductant. In *A. nidulans* (1), NADPH could work as electron donor but the

TABLE III

FERREDOXIN-DEPENDENT PHOTOREDUCTION OF NITRATE WITH WATER BY *Nostoc muscorum* PARTICLES

System	Nitrite formed (nmoles/mg chlorophyll)
Complete, light	710
Minus ferredoxin	96
Minus nitrate	0
Minus ADP, Pi	790
Minus particles	0
Plus CMU	3
Plus CMU, plus ascorbate/DPIP	750
Complete, dark	8

The complete system included in a final volume of 1 ml: 50 µmoles Tricine-KOH buffer, pH 7.7; 20 µmoles KNO_3; 10 µmoles $MgCl_2$; 2 µmoles ADP; 2 µmoles potassium phosphate; 0.1 mg *N. muscorum* ferredoxin; *N. muscorum* particles B containing 0.1 mg chlorophyll. Where indicated, 100 nmoles CMU, 14 mg ascorbate and 60 µg DPIP were added. The reaction was carried out under air at 25°C for 30 min. Light intensity, 20,000 lux.

addition of spinach NADP-reductase was necessary for the particles to perform the ferredoxin-dependent reduction of nitrate with this nucleotide. The fact that *N. muscorum* particles can utilize NADPH as electron donor, without the addition of extraneous diaphorase (Tables I and II), indicates the presence of a specific NADP-reductase that mediates the transfer of electrons from NADPH to the non-heme iron carrier.

When *Nostoc* particles were prepared by sonication a photo-

TABLE IV

PHOTOREDUCTION OF NITRATE TO NITRITE WITH WATER COUPLED TO STOICHIOMETRIC OXYGEN EVOLUTION BY PARTICLES OF *Nostoc muscorum*

System	Nitrite formed (nmoles)	Oxygen evolved (nmoles)
Complete, light	960	450
Minus ferredoxin	126	68
Minus nitrate	3	0
Plus CMU	4	0
Complete, dark	5	0

The complete system included in a final volume of 3 ml: 150 µmoles Tricine-KOH buffer, pH 7.7; 60 µmoles KNO_3; 30 µmoles $MgCl_2$; 0.3 mg *N. muscorum* ferredoxin; *N. muscorum* particles C containing 0.4 mg chlorophyll. Where indicated, 0.3 µmoles CMU was added. The reaction was carried out at 30°C for 10 min. Light intensity, 20,000 lux.

reduction of nitrate by water was observed. Electrons from water photolysis originating in photosystem II were transported through photosystem I and used for the ferredoxin-dependent reduction of nitrate catalyzed by the nitrate reductase bound to the photosynthetic particles. As Table III shows, the ferredoxin-dependent photoreduction of nitrate was prevented by CMU, a typical inhibitor of electron flow from photosystem II to photosystem I and could be restored after adding the ascorbate/DPIP couple. Nitrate reduction by subcellular particles was coupled to a stoichiometric oxygen evolution (Table IV). This demonstrates the direct participation of water as the ultimate source of

reducing power in the photoreduction of nitrate operated by the algal particulate system. Cell-free preparations from another blue-green alga, *A. nidulans*, behave in a similar way (P. Candau, C. Manzano and M. Losada, unpublished observation).

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