Nitrate reductase activity in isolated heterocysts of the cyanobacterium *Nostoc muscorum*

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The nitrate reductase, nitrate reductase apoprotein and nitrate reductase Mo-cofactor activities were measured in the cell-free extracts of isolated heterocysts and whole filaments of the cyanobacterium *Nostoc muscorum*. The nitrate reductase activity of N_2- and NO_3^--grown whole filaments was 3.25 and 7.14 nmol NO_2^-formed · min^{-1} · mg $protein^{-1}$, respectively. No nitrate reducatase activity was found in the extracts of isolated heterocysts. However, when the heterocyst extract was supplemented with nitrate reductase apoprotein from the whole filaments, a reconstituted nitrate reductase activity of 6.4 nmol NO_2^-formed · min^{-1} · mg $protein^{-1}$ was detectable. Therefore, it was concluded that nitrate reductase in *Nostoc muscorum* was localized in the vegetative cells, that the heterocysts lacked nitrate reductase activity due to the lack of apoprotein, and that the Mo-cofactor was present in heterocysts.

Cyanobacterium *Heterocyst* *Nitrate reductase* *Nitrate reductase apoprotein* *Nitrate reductase Mo-cofactor*
Nostoc muscorum

1. INTRODUCTION

Heterocysts of cyanobacteria are the sites of aerobic nitrogen-fixation and show various structural, physiological, biochemical and genetic modifications essential for their function [1,2]. In heterocystous cyanobacteria nitrogenase is localized in heterocysts and CO_2-fixation in vegetative cells [1,2]. Various enzymes involved in the reductive pentose phosphate cycle have been found to be absent in heterocysts [3]. The primary assimilation of ammonia, generated during N_2-fixation, occurs in heterocysts and fixed nitrogen is transferred to vegetative cells in the form of glutamine [4]. The CO_2-fixation occurs in vegetative cells and fixed carbon is transferred to heterocysts where it is utilized for generating reductant and energy for nitrogen-fixation [1]. Thus, there is a close interrelation between heterocysts and vegetative cells with regard to their carbon and nitrogen metabolism.

The specific activities of many of the nitrogen metabolizing enzymes of heterocysts and vegetative cells have been measured for comparison [2]. The glutamine synthetase [4,5], alanine dehydrogenase [5,6], glutamate dehydrogenase [5], glutamate oxaloacetate transaminase [5,6] and glutamate pyruvate transaminase [5] activities have been found to be present both in the heterocysts and in the vegetative cells. However, the nitrogenase was found to be localized in the heterocysts [7–12] and glutamate synthase in vegetative cells only [4,13]; see however [14,15]. Activities of enzymes responsible for both the synthesis and breakdown of cyanophycin, arginine-poly(aspartic acid) synthetase and cyanophycinase, respectively, have been reported to be much higher in the heterocysts than in the vegetative cells [16].

At present no information is available with regard to the enzymes of nitrate metabolism in the heterocysts of cyanobacteria. We have studied the nitrate reductase activity in isolated heterocysts of *Nostoc muscorum* and show here that the nitrate reductase activity is absent in heterocysts due to the lack of nitrate reductase apoprotein.

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2. MATERIALS AND METHODS

Nostoc muscorum was grown axenically in nitrogen-free and NO_3^--supplemented Chu-10 medium [17] as in [18]. *Escherichia coli* strain W was grown aerobically in Luria broth [19] supplemented with 1 mmol · dm^{-3} sodium molybdate.

Heterocysts were isolated from the exponentially growing filaments of *N. muscorum* as in [20]. Such heterocyst preparations had a glutamine synthetase biosynthetic activity of 174 nmol product formed · min^{-1} · mg $protein^{-1}$ and a glutamine synthetase transferase activity of 3840 nmol product formed · min^{-1} · mg $protein^{-1}$ (both assayed as in [21]). Vegetative cells were less than 5% of the total.

The protein estimations were made as in [22]. In vitro nitrate reductase activity was measured as in [23].

Mo-cofactor preparation from *E. coli* was essentially the same as in [24]. To prepare Mo-cofactor from heterocysts, heterocysts were isolated from cultures grown in Mo-containing medium, washed and resuspended in Tris buffer (50 mmol · dm^{-3}, pH 7.5) containing 100 mmol · dm^{-3} NaCl, 300 mmol · dm^{-3} sucrose, 1 mmol · dm^{-3} EDTA and 5 mmol · dm^{-3} $MgCl_2$. The cell-free extract was then prepared by passage through a French Pressure Cell at 110 MPa followed by centrifugation at 30 000 × *g* for 20 min at 4°C. The supernatant was used as a source of Mo-cofactor for complementation analysis.

For preparation of Mo-cofactor-free nitrate reductase (nitrate reductase apoprotein), 200 mm^3 cell-free extract, prepared as above, incubated with 200 mm^3 molybdate–GSH solution (10 mmol · dm^{-3} GSH and 5 mmol · dm^{-3} sodium molybdate in 100 mmol · dm^{-3} sodium acetate buffer, pH 4.5). This brought the pH of the reaction mixture to 4.8. After 30 s incubation at 30°C (acid incubation) the pH was adjusted to 7.2 by adding 200 mmol · dm^{-3} K_2HPO_4 and incubated for 15 min at 30°C (neutral incubation).

Reconstituted nitrate reductase activity was measured by mixing equal volumes of the nitrate reductase apoprotein (cofactor-free nitrate reductase) and the Mo-cofactor. This mixture was incubated for 10 min at 30°C to achieve complementation and then nitrate reductase activity was assayed as mentioned above.

3. RESULTS AND DISCUSSION

The aim of this study was to establish whether or not the heterocysts contain nitrate reductase activity. For this we isolated heterocysts from *N. muscorum* filaments and measured the nitrate reductase activity in the cell-free extracts. For comparison, data were also obtained on nitrate reductase activity in the cell-free extracts of *N. muscorum* filaments grown in nitrogen-free (filaments containing both heterocysts and vegetative cells) and in nitrate media (filaments containing vegetative cells only).

The nitrate reductase activity was undetectable in the extracts of heterocysts while a nitrate reductase activity of 3.25 and 7.14 nmol NO_2^- formed · min^{-1} · mg $protein^{-1}$ was observed in the extracts of filaments grown in nitrogen-free and nitrate media, respectively (table 1). The higher level of nitrate reductase activity in nitrate-grown filaments of *N. muscorum* was consistent with earlier reports [25,26]. However, the absence of nitrate reductase in heterocysts, but its presence in whole filaments containing vegetative cells and heterocysts, was noteworthy. It suggested that nitrate reductase activity was localized in the vegetative cells only.

We further investigated whether the lack of nitrate reductase activity in heterocysts was due to the absence of nitrate reductase Mo-cofactor, nitrate reductase apoprotein or both. First, nitrate reductase apoprotein (Mo-cofactor-free nitrate reductase), prepared from isolated heterocysts, was supplemented with increasing amounts of Mo-cofactor prepared from *E. coli,* but nitrate reductase activity could not be restored. This indicated the absence of nitrate reductase apoprotein in the heterocysts. It could be argued that the Mo-cofactor from *E. coli* may not be compatible with the nitrate reductase apoprotein from *N. muscorum*. However, this is not so. The nitrate reductase Mo-cofactor from *E. coli* reconstituted nitrate reductase activity when added to the apoprotein preparations from *N. muscorum* filaments, grown in nitrogen-free or nitrate medium, suggesting that the Mo-cofactor of *E. coli* was compatible with the apoprotein of nitrate reductase from *N. muscorum*.

Secondly, the nitrate reductase apoprotein preparations from whole filaments of *N. muscorum,* grown in nitrogen-free medium, were sup-

Table 1

Nitrate reductase activity in cell-free extracts of isolated heterocysts and whole filaments of *Nostoc muscorum* grown in nitrogen-free and nitrate medium

Enzyme source	Activity (nmol NO_2^- formed · min^{-1} · mg $protein^{-1}$)
N_2-grown *N. muscorum* extract	3.25
Nitrate-grown *N. muscorum* extract	7.14
Isolated heterocyst extract	not detectable
Apoprotein from N_2-grown *N. muscorum*	not detectable
Apoprotein from nitrate-grown *N. muscorum*	not detectable
Isolated heterocyst extract (Mo-cofactor) supplemented with apoprotein preparation from N_2-grown *N. muscorum*	6.25[a]
Isolated heterocyst extract (Mo-cofactor) supplemented with apoprotein preparation from nitrate-grown *N. muscorum*	6.39[a]

[a]Specific activity expressed per mg protein of the heterocyst extract. The amount of apoprotein preparations used were similar in both cases (protein concentration 2 mg · cm^{-3})

plemented with Mo-cofactor prepared from isolated heterocysts. Under such conditions a reconstituted nitrate reductase activity of 6.25 nmol NO_2^- formed · min^{-1} · mg $protein^{-1}$ was detected. A similar level of reconstituted nitrate reductase activity was detected when Mo-cofactor preparations from heterocysts were complemented with nitrate reductase apoprotein from whole filaments of *N. muscorum* grown in nitrate medium (table 1). The fact that the filaments grown in nitrogen-free medium had a lower nitrate reductase activity than those grown in nitrate medium while their apoprotein preparations showed similar levels of reconstituted nitrate reductase activity when complemented with similar amounts of Mo-cofactor from isolated heterocysts, suggested that the Mo-cofactor may have been limiting in the vegetative cells of *N. muscorum* grown in nitrogen-free medium (nitrogen-fixing condition) and that heterocysts had a higher level of Mo-cofactor than vegetative cells. The higher nitrate reductase activity in filaments of *N. muscorum* grown in nitrate medium therefore may have been due to the increased level of Mo-cofactor resulting from the lack of competition, between nitrate reductase and nitrogenase, for the Mo-cofactor.

It should be mentioned that these apoprotein preparations did not show any nitrate reductase activity unless supplemented with Mo-cofactor preparations (from *E. coli,* isolated heterocysts or whole filaments). Furthermore, addition of Mo or ferredoxin, alone or together, did not restore nitrate reductase activity in the apoprotein preparations. This ruled out the possibility that reconstituted nitrate reductase activity in apoprotein preparations supplemented with Mo-cofactor from heterocysts may have been due to Mo or ferredoxin rather than to the Mo-cofactor.

It is clear from the above observations that the nitrate reductase Mo-cofactor is present in heterocysts and that the absence of nitrate reductase in heterocysts is due to the absence of nitrate reductase apoprotein. The presence, in heterocysts, of Mo-cofactor which can complement the apoprotein from vegetative cells and show reconstituted nitrate reductase activity is consistent with the view expressed earlier [25,27] that the Mo-cofactor of nitrate reductase in *N. muscorum* may be a precursor for the Fe-Mo-cofactor of nitrogenase.

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