Induction of Nitrate Reductase Activity by Arginine in the Filamentous Cyanobacterium *Oscillatoria chalybea*

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In *Oscillatoria chalybea,* as in other cyanobacteria, nitrate reduction depends directly on photosynthetic activity. Hence, nitrate reduction occurs only in the light and appears inhibited when photosynthesis is inhibited by the herbicide DCMU. Growth of *Oscillatoria* cells is possible on a variety of exogenous nitrogen sources in the medium and appears largely independent on the type of nitrogen source. However, if citrulline is the exogenous nitrogen source or if no exogenous nitrogen source is given in the medium, growth appears almost fully inhibited. Nitrate reductase activity measured in French-press particles of nitrate-grown cells is dependent on the age of the culture with maximum nitrate reductase activity being reached on the 5th day. Thereafter activity decreases steeply to less than 20% of the maximal activity within 10 days. Besides the growth stage it is the type of exogenous nitrogen source used in the medium which is important for the development of nitrate reductase activity. It appears that in the presence of nitrate, nitrite and arginine, nitrate reductase activity is induced whereas in the presence of ammonia or amino acids like alanine nitrate reductase activity is not induced, as already reported in the literature. Nitrate reductase is also induced if arginine *and* ammonia are simultaneously offered as exogenous nitrogen source. Arginine metabolism in *Oscillatoria* cells is characterized by the fact that thylakoid preparations of *Oscillatoria* catalyze the transformation of arginine to give ornithine and ammonium. The arginine-metabolizing enzyme differs from the usual arginine-induced arginase. The enzyme seems to be constitutive, not manganese-dependent, exhibiting an approximately 5 times higher substrate affinity to arginine than the known arginase. In the present paper we propose that in *Oscillatoria* it is arginine which induces the synthesis of nitrate reductase.

Introduction

In cyanobacteria there exists a very close relationship between photosynthetic and respiratory electron transport which is due to the fact that both processes occur in the same, namely in the thylakoid membrane. Moreover, in contrast to the eukaryotic condition the entire nitrate reducing enzyme system is also localized in the thylakoid membrane [1]. Thus, the structure/function relationship in the thylakoid membrane of these organisms is different to that in the higher plant membrane and a particularly close interrelationship between photosynthesis, respiration and nitrogen metabolism in particular nitrate reduction seems evident. Cyanobacteria are able to use nitrate, nitrite and ammonium as an inorganic nitrogen source [2]. It appears that nitrate uptake as well as the synthesis of nitrate reductase are inhibited in the presence of ammonium [1, 3]. As the inhibition of glutamine synthetase by methionine sulfoximine (MSX) relieves this ammonium-induced inhibition of nitrate uptake as well as that of the enzyme synthesis, it is generally assumed that a metabolite derived from the incorporation of ammonium is the inhibitor [4].

Besides inorganic nitrogen compounds cyanobacteria successfully use organic nitrogen compounds. In an earlier publication we were able to show that, although *Oscillatoria chalybea* grows on most any amino acid as the sole nitrogen source in the growth medium, only the basic amino acid arginine leads to the synthesis of nitrate reductase [5]. In the present paper we are able to show that the extent of nitrate reduction in *Oscillatoria chalybea* not only depends on the growth stage of the organism but also on the type of nitrogen source available in the culture medium. These studies lead to a new hypothesis for the mechanism of the regulation of nitrate reductase synthesis.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-N,N′-dimethylurea; MSX, methionine sulfoximine.

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Materials and Methods

The filamentous cyanobacterium Oscillatoria chalybea was obtained from the algal collection of the University of Göttingen and grown on clay plates of 11 cm in diameter, which were just submerged in the respective culture medium in large petri dishes. The culture medium composition contained 3.5 mM K_2HPO_4, 0.6 mM $MgSO_4$, 42 μM $CaCl_2$, 12 μM FeEDTA, 16 μM H_3BO_3, 3.5 μM $ZnSO_4$, 0.7 μM $MnSO_4$, 0.5 μM Na_2MoO_4, 0.3 μM $CoCl_2$, 0.2 μM $CuSO_4$. If grown on different nitrogen sources the respective concentration of the nitrogen compound in the medium was 10 mM $NaNO_3$, 5 mM $NaNO_2$, 10 mM $(NH_4)_2SO_4$, 5 mM arginine, 10 mM alanine, 5 mM agmatine, 10 mM ornithine, 6.25 mM citrulline or 10 mM aspartic acid. If a combination of two nitrogen sources was used, only half of the above given concentration for each compound was used. The pH of the medium was 8.1. The cyanobacteria were grown at 26 °C in a light/dark cycle of 14 h light/10 h dark. Light intensity was 1000 to 1500 lux.

Isolation of individual cells: The filaments were centrifuged at 500 × *g* and suspended in 10 mM potassium-phosphate buffer at pH 8.0. By means of a homogenizer (B. Braun, Melsungen AG) the cells were separated from the filament organization. The cells were washed twice with 10 mM potassium-phosphate buffer and resuspended in the same buffer.

Preparation of French-press particles: Cells obtained, as described above, were broken up by two successive French-press (American Instrument Company) treatments at 10,000 psi. Still intact cells were spun down by a short time centrifugation at 500 × *g*.

Preparation of thylakoid preparations: The French-press preparation was centrifuged at 95,000 × *g* for 40 min. The sedimented thylakoids were resuspended in 10 mM potassium-phosphate buffer.

Ammonium determination: Ammonium determinations were carried out according to the Boehringer procedure [6]. The assay contained in 3 ml: 200 μmol triethanolamine buffer pH 8.6, 300 μmol α-ketoglutarate, 3 μmol ADP, 0.48 μmol NADH and 0.1–0.3 ml of the solution to be analysed. Extinction of the assays was measured at 334 nm and 5 μl glutamate dehydrogenase from Boehringer were added. The induced extinction decrease was measured after 10 min. After another addition of 5 μl glutamate dehydrogenase the extinction decrease was measured again after 10 min. The ammonia concentration was determined *via* the extinction coefficient of NADH at 334 nm (= 6.180 $M^{-1}\,cm^{-1}$).

Chlorophyll determination: Chlorophyll was determined according to Schmid [7].

Nitrate determination: The determination of the nitrate concentration was carried out according to Cawse [8].

Nitrite determination: Nitrite was determined according to Snell and Snell [9].

Protein concentrations were determined according to Lowry *et al.* [10].

Nitrate reductase activity was carried out according to the procedure by Herrero *et al.* [11]. The reaction assay contained in 1 ml: 100 μmol $NaHCO_3/Na_2CO_3$-buffer pH 10.5, 10 μmol KNO_3, 4 μmol methylviologen and 10 μmol sodium dithionite in 0.1 ml 0.3 M $NaHCO_3$. The assay was incubated at 30 °C for 10–15 min.

Results

In cyanobacteria nitrate reductase activity *in vivo* is directly linked to photosynthesis as reducing equivalents from photosynthetic electron

Table I. Nitrate consumption and production of nitrite and ammonia in intact cells of *Oscillatoria chalybea*.

Condition	14-Day old cells		6-Day old cells			
	Light	Light + MSX	Light	Light + MSX	Light + DCMU	Dark
	[μmol × mg chlorophyll^{-1} × h^{-1}]					
NO_3^--Consumption	1.0	0.94	1.6	0.79	0.0	0.0
NO_2^--Production	0.98	0.66	0.57	0.22	0.0	0.0
NH_4^+-Production	–	0.43	–	0.52	0.0	0.0

transport such as reduced ferredoxine are used for the nitrate and nitrite reductase reaction. Correspondingly, also in *Oscillatoria chalybea* nitrate reduction is only observed in the light and is inhibited in the light by the photosystem II-inhibiting herbicide DCMU (Table I). Cells of *Oscillatoria chalybea* are able to grow on a large variety of exogenous nitrogen sources. Not much difference in the growth rate is observed if the cells are grown on nitrate, ammonium or on amino acids as nitrogen source in the medium. Only if the cells are grown on citrulline or if an exogenous nitrogen source in the medium is omitted, growth appears to be severly inhibited. Fig. 1 shows growth curves of nitrate-grown *Oscillatoria chalybea* cultures with reference to the protein and chlorophyll content. The level of nitrate reductase activity in *Oscillatoria* depends strongly on the growth stage of the culture (Fig. 2). It appears that the enzyme activity of French-press particle preparations reaches a steep maximum within 5 to 6 days and then decreases to a steady state level which is less than

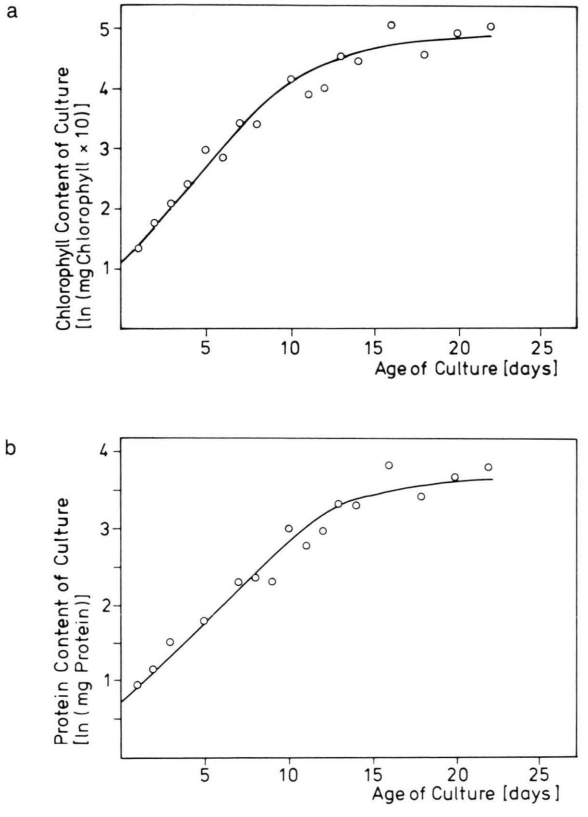


Fig. 1. Growth curves of an *Oscillatoria chalybea* culture grown on nitrate measured in a) as increase in chlorophyll content and in b) as increase of protein content as a function of time.

Fig. 2. Nitrate reductase activity in nitrate-grown *Oscillatoria chalybea* in dependence on the age of the culture.

20% of the maximal value (Fig. 2). Due to the necessary culture conditions on clay plates (see Materials and Methods) one is always confronted to a lack of cell material with this cyanobacterium, which in the past made us use dense 3 weeks-old cultures in which as it appears now not much nitrate reductase activity is present. In addition to this precise definition of the growth stage for optimal nitrate reductase activity it is the chemical nature of the nitrogen source in the culture that matters. In Table II it is seen that a number of compounds induce nitrate reductase activity. These are on the one hand nitrate and nitrite, already known from the literature [12], and on the other hand, as shown here, arginine and citrulline. Other amino acids as alanine or ornithine do not induce nitrate reductase activity nor does ammonium (Table II) which is interpreted in the literature as beeing due to a metabolite resulting from ammonium incorporation [4]. This suggests that nitrate reductase is induced by arginine. This is supported by the observation that nitrate reductase activity in arginine- or nitrate-grown cultures appears nearly simultaneously (Fig. 3). If the culture is grown on arginine plus ammonium sulfate, nitrate reductase

Table II. Nitrate reductase activity of French-press particle preparations of *Oscillatoria chalybea*, grown on different exogenous nitrogen sources in the culture medium.

Nitrogen source	6-Day old cells [µmol NO_2 produced × mg chlorophyll^{-1} × h^{-1}]	14-Day old cells
Nitrate	61.2	16.2 (0.324)
Nitrite	44,0	14.3
Ammonium	10.0	2.0 (0.040)
Arginine	124.7	47.1
Citrulline	132.7	32.2
Alanine	6.9	1.2
Ornithine	24.8	9.1
Agmatine	43.7	2.1
Aspartic acid	41.7	6.6
No source in the medium ("N-free")	41.0*	49.2* (0.147)

* Cells are chlorotic and chlorophyll may not be the suitable reference in this culture. In all other cultures the ratio of chlorophyll to dry weight is the same and found to be 0.02. It should be noted that the chlorophyll/protein ratio in all cultures appears to be approximately the same. Values in brackets are with reference to dry weight.

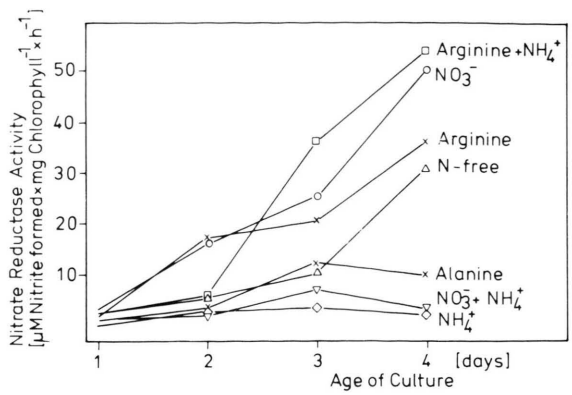


Fig. 3. Nitrate reductase activity in *Oscillatoria chalybea*, grown on different exogenous nitrogen sources in the medium, as a function of the age of cultures.

activity is induced from the second day onward and reaches the same maximal value on the 4th day as a nitrate-grown culture (Fig. 3). In a culture with nitrate plus ammonium, however, no nitrate reductase activity is induced (Fig. 3). It should be noted that nitrate reductase activity is observed in a 3 day old culture in which an exogenous nitrogen source in the medium has been omitted (Fig. 3). Although having no heterocysts, *Oscillatoria* can fix atmospheric nitrogen with a low rate (experiments not shown here). That arginine might be the real inductor of nitrate reductase is strengthend by experiments, in which addition of arginine in the stationary phase of growth of a nitrate culture (Fig. 1), where nitrate reductase activity is low, leads to a substantial built up of nitrate reductase (Table III). The same observation is made if in an ammonium sulfate-grown culture in the stationary growth phase after 17 days 10 mM arginine is added and if cells are analysed for nitrate reductase activity 4 days later (Table III).

The fate of arginine in *Oscillatoria chalybea* seems not to be exclusively linked to an arginase. In this cyanobacterium as in others presence of arginine in the medium induces synthesis of arginase. Citrulline and ornithine also induce in *Oscillatoria*

Table III. Effect of arginine addition to nitrate-grown cultures in the stationary growth stage and to ammonium sulfate-grown cultures on the induction of nitrate reductase activity in the filamentous cyanobacterium *Oscillatoria chalybea*.

Cultures grown on	Nitrate reductase activity [µmol nitrite formed × mg chlorophyll^{-1} × h^{-1}]
Nitrate for 25 days	6.2
Nitrate for 25 days but after 21 days addition of 10 mM arginine	18.4
Ammonium for 21 days	0.5
Ammonium for 21 days but after 17 days addition of 10 mM arginine	18.9

Table IV. Properties of the arginine-metabolizing enzyme and of the inducible arginase in the filamentous cyanobacterium *Oscillatoria chalybea*.

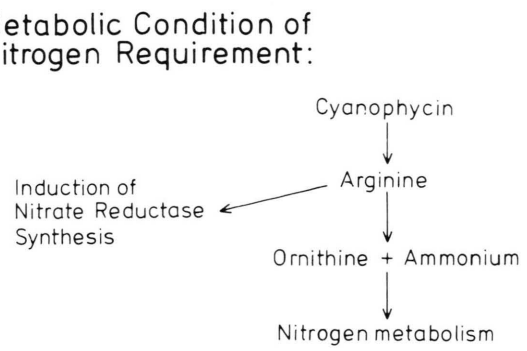
	Arginase	Arginine-metabolizing enzyme
K_m (arginine)	3 mM	0.5 mM
Substrate saturation	10 mM	2.0 mM
Activity [μmol ornithine formed × mg chlorophyll^{-1} × h^{-1}]		
without addition of Mn^{2+}	3.0	0.8
with addition of Mn^{2+}	45.0	0.8
Occurrence	inducible by arginine, ornithine and citrulline	constitutive

arginase, the activity of which depends on manganese in the assay and which exhibits a substrate affinity for arginine corresponding to a K_m of 3.0 mM (Table IV). It appears however, that *Oscillatoria* owns in addition a constitutive arginine metabolizing enzyme which does not depend on manganese and which exhibits a much higher affinity for arginine corresponding to a low K_m of 0.5 mM (Table IV). The enzyme catalyzes a reaction in which the reaction products are ornithine and ammonium.

Discussion

In the stationary growth stage, which is reached in two to three weeks old cultures, *Oscillatoria chalybea* grown on nitrate exhibits the following metabolic activities: In low light conditions, which correspond to the intensities under which the organism is able to grow, photosynthetic electron transport between the photosystems is disrupted, although the measureable photosystem I and photosystem II activities are high. Hardly any electrons from water arrive at P700 [5]. The capacity present for photosystem II-reactions exceeds under these culture conditions that for photosystem I [5] but nitrate reductase activity as shown in the present paper is low and corresponds to 1/5 of the activity in the exponential phase (Fig. 1). Under these conditions however, a photosystem II mediated H_2O_2/O_2 cycle is operative suggesting the occurrence of an energy producing oxidative process [13]. Under these growth conditions in the stationary growth stage, the organism is full of cyanophycin as shown by electron microscopy [5].

Oscillatoria chalybea like any other cyanobacterium can use nitrate, nitrite or ammonia as an organic nitrogen source. Besides this, most any amino acid can be used as nitrogen source under heterotrophic growth conditions. But amongst these amino acids only growth on arginine or its derivative citrulline leads to nitrate reductase activity in the cells (Table II). We therefore think that arginine is a (the) inductor for the synthesis of nitrate reductase. The experimental observations on the induction and repression of nitrate reductase reported in the literature clearly show, that uptake of nitrate into the cells as well as synthesis of nitrate reductase are inhibited in the presence of ammonium [3]. As this inhibition is not observed in the presence of methionine sulfoximine, an inhibitor of glutamine synthetase, the inhibitor is searched in the region of a metabolic compound resulting from ammonium incorporation [4]. Our experiments show that addition of arginine to a nitrate grown culture in the stationary phase, in which nitrate reductase is low (Fig. 2), leads to the induction of nitrate reductase activity (Table III). Most remarkingly is that in an ammonium sulfate-grown culture in which nitrate reductase is repressed addition of arginine also leads to an induction of nitrate reductase (Fig. 3, Table III). We therefore propose for the induction of nitrate reductase in *Oscillatoria* a reaction sequence in which *via* the mobilization of the internal storage product cyanophycin (poly-arginyl-aspartate) ar-



Scheme: Proposed reaction sequence leading to the induction of nitrate reductase in the filamentous cyanobacterium *Oscillatoria chalybea*.

ginine is produced which then induces nitrate reductase synthesis (Scheme). The reason for this might be that when cells from a stationary culture are transfered to fresh growth medium and when these cells start to grow, the nitrate reductase activity present is insufficient to cover the needs for nitrogen. Therefore, cyanophycin is decomposed which *via* an arginine-mediated induction leads to an enhanced nitrate reductase synthesis. Thereafter the nitrogen storage pool (cyanophycin) is refilled and nitrate reductase activity falls to the level sufficient for the cells in this growth stage. The same mechanism explains the induction of nitrate reductase synthesis when no exogenous nitrogen source is present in the medium. In the presence of ammonia or in the presence of organic nitrogen no lack of nitrogen, hence no decomposition of cyanophycin and therefore no induction of nitrate reductase occurs. Presence of arginine in our experiments (shown in Table II, III etc.) might simulate a lack of nitrogen thus leading to the synthesis of nitrate reductase.

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