Further Studies on the Induction of Nitrate Reductase by Arginine in the Filamentous Cyanobacterium *Oscillatoria chalybea*

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In an earlier publication we reported on the role of arginine for the development of nitrate reductase activity in cells of the filamentous cyanobacterium *Oscillatoria chalybea* (Bednarz and Schmid, Z. Naturforsch. **46c,** 591 (1991)). In the present paper we present further evidence that arginine is the natural inducer for nitrate reductase activity. Thus, we show that the induction is regulated by transcription, probably related to the apoprotein or the molybdenum-cofactor. We also examined the influence of arginine on nitrate reductase activity in the filamentous cyanobacterium *Anabaena* PCC 7120. In contrast to *Oscillatoria chalybea* this cyanobacterium forms heterocysts and shows nitrogen fixation activity. Like in *Oscillatoria chalybea* nitrate reductase activity in *Anabaena* PCC 7120 is stimulated in the presence of arginine as the sole nitrogen source. However, this stimulation is limited to an early growth stage. Subsequently, nitrogen fixation activity appears and nitrate reductase activity decreases.

Introduction

Assimilatory nitrate reduction in cyanobacteria is a regulated process. However, the mechanism of this regulation remains unknown. In all cyanobacteria studied so far ammonium seems to repress the expression of nitrate reductase activity. This influence is observed when ammonium is metabolized through glutamine synthetase [2]. If this enzyme is inhibited by MSX (methionine sulfoximine), no repression takes place. There are different views on the induction of nitrate reductase activity by nitrate: First, according to Herrero *et al.* [3] *de novo* synthesis of nitrate reductase takes place in filamentous, heterocyst-forming cyanobacteria like *Nostoc* and *Anabaena* only after transfer to nitrate containing medium. On the other hand in unicellular cyanobacteria like *Synechococcus* nitrate is not obligatory since induction of nitrate reductase takes also place after transfer to media with no bound nitrogen. This has also been shown for the filamentous non heterocyst-forming cyanobacterium *Oscillatoria chalybea* [1]. Second, according to Bagchi *et al.* [4] and Avissar [5] there is a derepression of nitrate reductase activity in filamentous nitrogen-fixing cyanobacteria when cells are grown on molecular nitrogen. They have shown, that nitrate is not required for *de novo* synthesis of the free apoprotein, but is needed for the *de novo* synthesis of the molybdenum-cofactor.

We have recently proposed that induction of nitrate reductase activity occurs when the cells need nitrogen for growth. The response to this condition leads to a mobilization of arginine which in the form of cyanophycin serves as nitrogen storage. And it was assumed that this arginine induced the induction of nitrate reductase activity [6].

In the present paper we show, that induction of nitrate reductase activity indeed occurs as response to nitrogen deficiency during growth of the cells. It looks as if an arginine mobilization is correlated with the synthesis of nitrate reductase apoprotein or molybdenum cofactor. This synthesis is shown to be regulated on the transcriptional level. Furthermore, we analyzed the induction process in *Anabaena* PCC 7120, a nitrogen fixing cyanobacterium in order to elucidate the importance of nitrogen fixation for the induction of nitrate reductase.

Materials and Methods

Oscillatoria chalybea was cultivated under the previously specified conditions [1]. *Anabaena* PCC 7120 was grown in medium BG 11 containing 0.25 mM $CaCl_2$, 0.18 mM K_2HPO_4, 0.3 mM $MgSO_4$, 2.7 µM Na-EDTA, 0.19 µM Na_2CO_3, 0.03 mM

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Ammonium Fe-Citrate, 0.05 mM H_3BO_3, 0.2 μM $MnCl_2$, 0.8 μM $ZnSO_4$, 0.1 μM Na_2MoO_4, 0.3 μM $CuSO_4$, 0.5 μM $CoSO_4$. If grown on different nitrogen sources the respective concentration of the nitrogen compound in the medium was 10 mM $NaNO_3$, 2 mM $(NH_4)_2SO_4$ or 5 mM L-arginine. When $(NH_4)_2SO_4$ was added the concentration of K_2HPO_4 was increased ten-fold to prevent acidification of the medium. The cultures were grown photoautotrophically at 26 °C in a stream of 0.5% CO_2 in air.

Determinations of ammonium, chlorophyll, nitrate, nitrite and nitrate reductase activity were performed as described before [1].

For studying the effect of nitrogen deficiency, cultures of *Oscillatoria chalybea* were grown on 1 mM $NaNO_3$ as sole nitrogen source. The concentration of nitrate, nitrite and ammonium in the medium as well as the nitrate reductase activity of the cells were determined over a period of 18 days.

In order to examine the mechanism of induction of nitrate reductase activity, we added 10 mM arginine to nitrate-grown cultures of *Oscillatoria chalybea*. This addition of arginine causes an increase in nitrate reductase activity of the cells, as demonstrated earlier [1, 6]. To some of the cultures the antibiotics rifampicin or chloramphenicol were added at the concentration of 50 $\mu g \times ml^{-1}$ medium.

Arginine-metabolizing activity: The reaction assay contained in 3 ml: 200 μmol Tricine pH 8.5, 3 μmol L-arginine, 0.5–1.0 ml thylakoid preparation as described earlier [1] corresponding to 30–50 μg chlorophyll. The assay was incubated at 30 °C for 5 h. The reaction was stopped by adding 0.1 ml 2 N H_2SO_4. Then the assay was centrifuged at 5,000 × *g* for 5 min and the clear supernatant was used for the analysis of reaction products, *i.e.* citrulline, ornithine and ammonia.

Citrulline determination: Citrulline was determined according to Prescott and Jones [7]. The color mix used in the assay consists of a mixture of two parts of 0.5% antipyrine in 50% (v/v) sulfuric acid and one part of 0.8% diacetyl-monoxime in 5% (v/v) acetic acid. 1 ml of this solution was added to 2 ml of the sample and this reaction mixture was incubated at 100 °C for 20 min. After cooling the absorbancy at 466 nm was read.

Ornithine determination: Ornithine was determined according to Ratner [8]. The reagent, prepared fresh daily, was made by dissolving 375 mg ninhydrin and 55 mg hydrindantin in a mixture of 6 ml 6 M H_3PO_4 and 9 ml of glacial acetic acid by heating to boiling for a few minutes. 0.5 ml of this reagent were mixed with 0.5 ml of the sample to be analyzed and 0.75 ml glacial acetic acid. The mixture was heated to 100 °C for 30 min. After cooling to room temperature 1.5 ml glacial acetic acid was added and the absorbancy was read at 515 nm.

Results

Nitrate reduction in *Oscillatoria chalybea* is accompanied by excretion of nitrite into the medium (Fig. 1). We never observed excretion of ammonium. When cells were grown on 10 mM nitrate for 4 weeks, the nitrate concentration decreased to less than 3 mM whereas the nitrite concentration increased to more than 3 mM.

Thus, not all the nitrate-nitrogen reduced by nitrate reductase directly serves as a nitrogen source to the cells. When cells were grown on 1 mM nitrate nitrite excretion was also observed (Fig. 2). But by the time when nitrate concentration had decreased to zero this nitrite was also assimilated and used as nitrogen source.

The induction of nitrate reductase activity in a cell culture showed the same behaviour regardless whether grown on 1 mM nitrate (Fig. 2) or on 10 mM nitrate [1]. Within the first days nitrate reductase activity increased up to a factor of five and then decreased, reaching the initial minimal rate after ten days. The observed increase in nitrate re-

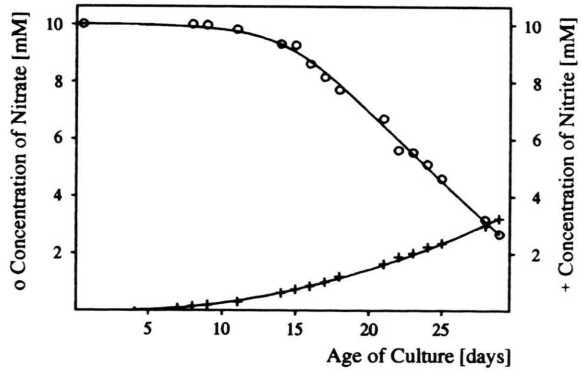

Fig. 1. Changes of nitrate and nitrite concentration in the medium during growth of *Oscillatoria chalybea* on nitrate as the sole nitrogen source.

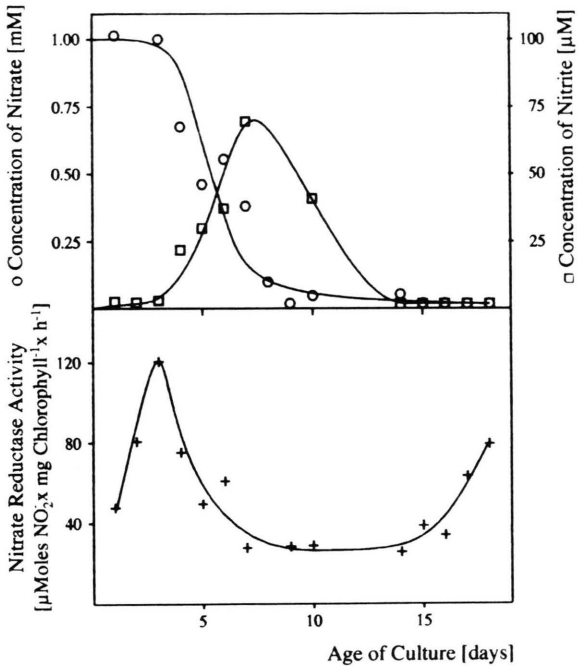

Fig. 2. Nitrate and nitrite concentrations in the culture medium. The upper part of the figure shows changes in nitrate and nitrite concentration during growth of *Oscillatoria chalybea* on 1 mM nitrate. The lower part shows the time course of nitrate reductase activity of the cells.

ductase activity during the first days is clearly accompanied by an increase in arginine-metabolizing activity (Table I). As shown by us earlier, cells grown on nitrate contain the storage product cyanophycin [6]. Therefore, arginine, formed by cyanophycin degradation, seems to be metabolized to ornithine and ammonia in a relation of

Table I. Arginine metabolizing activity in different growth stages of nitrate-grown cultures of *Oscillatoria chalybea*.

Age of culture	Ornithine	Citrulline	Ammonia	Ammonia / ornithine
	(nmoles × mg chlorophyll^{-1} × h^{-1})			
1 day	180	0	320	1.8
3 days	640	0	1,570	2.5
5 days	570	0	1,210	2.1
7 days	440	0	1,130	2.6
12 days	160	80	700	4.4
14 days	210	240	990	4.8

The assay contained in 3 ml thylakoid preparation [1] corresponding to 30 to 50 μg chlorophyll and 3 μmol arginine.

1 to 2 (Table I), an enzyme activity which supplies the cells with ammonium-nitrogen. In the later growth stage arginine is metabolized to citrulline and ammonia (1 to 1) by an arginine-deiminase, a reaction which also provides the cells with energy in form of ATP (data not shown).

However, from that moment onward when all the combined nitrogen in the medium is used up nitrate reductase activity increased again (Fig. 2).

In following experiments we addressed the question what kind of regulation is involved in the control of nitrate reductase activity. We used cultures which were grown on nitrate for 16 days and added to some of these cultures 10 mM arginine. As has been shown before [1] this addition causes a stimulation of nitrate reductase activity of the cells (Table II). This stimulation was suppressed, when

Table II. Influence of the antibiotics rifampicin and chloramphenicol on nitrate reductase activity in *Oscillatoria chalybea*.

Added substance	Nitrate reductase activity (μmol nitrite formed × mg chlorophyll^{-1} × h^{-1})
No addition	12.0
Rifampicin	5.9
Chloramphenicol	7.2
Arginine	58.7
Arginine + Rifampicin	10.8
Arginine + Chloramphenicol	12.5

Cells were grown on nitrate as the sole nitrogen source for 16 days. Three days before the determination of enzyme activity arginine was added to some cultures. At the same time the antibiotics rifampicin or chloramphenicol were added to the cultures as indicated.

there was a simultaneous addition of the antibiotics rifampicin or chloramphenicol. Presence of these antibiotics alone, without addition of arginine caused only a slight decrease in nitrate reductase activity (Table II).

For comparison we investigated the regulation of the process in *Anabaena* PCC7120 and three chlorate resistent mutants of this filamentous heterocyst-forming cyanobacteria. Whereas in *Oscillatoria chalybea* induction of nitrate reductase activity occurred similarly in presence of nitrate or arginine as the sole nitrogen source, the induction of this enzyme activity in *Anabaena* PCC7120 differed depending on the nitrogen source. After the transfer to nitrate containing medium, nitrate reductase activity increased reaching a maximum after five hours. In the presence of arginine as sole nitrogen source the increase of enzyme activity lasted only two and a half hours and decreased then to the initial value (Fig. 3).

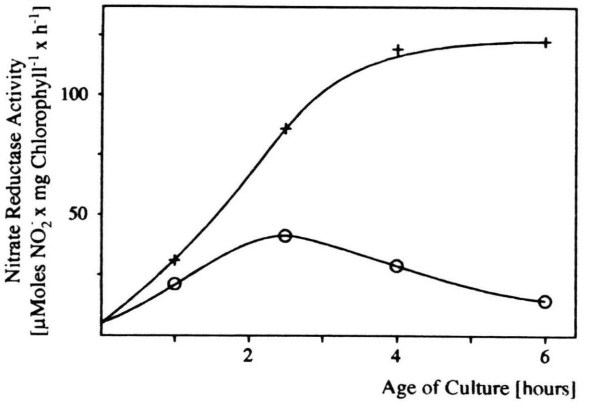

Fig. 3. Nitrate reductase activity measured in French-press preparations obtained from *Anabaena* PCC7120 cultures after the transfer to a medium with nitrate (+) or arginine (○) as the sole nitrogen source.

The results presented in Table III clearly show, that there is an identical induction of nitrate reductase activity within two and a half hours after transfer to nitrate, arginine or nitrogen-free medium but no induction after transfer to the ammonium containing medium. This is true for the wild type and for one of the mutants. In the other two mutants no induction of nitrate reductase activity has been observed.

Discussion

A peculiarity of assimilatory nitrate reduction of *Oscillatoria chalybea* is the excretion of nitrite into the medium which is also observed in *Oscillatoria rubescens* [9]. This excretion seems to be caused by the unusual low affinity for nitrite of nitrite reductase in *Oscillatoria chalybea*. The K_m (nitrite) for this enzyme was estimated to be 1.1 mM meaning that the affinity for nitrite is more than ten times lower than in *Synechococcus* PCC6301 or *Anabaena* PCC7119 [10]. It should be noted that we did not observe a change in nitrite reductase activity after the transfer of the cells to nitrate medium. Hence, the extent of nitrite reduction is not regulated by the enzyme concentration but by nitrite concentration and the nitrite concentration in the cell is regulated by nitrate reductase activity. This means that nitrate reduction in *Oscillatoria chalybea* is regulated by nitrate reductase activity itself.

By use of the antibiotics rifampicin and chloramphenicol we were able to show, that nitrate reductase is under transcriptional control. This control may affect the apoprotein or the molybdenum cofactor which is needed for enzyme activity. Induction of nitrate reductase occurs under nitrogen limitation. As we have shown before [1], nitrogen limitation leads to cyanophycin degradation and

Table III. Nitrate reductase activity in French-press preparations of *Anabaena* PCC7120 and the three chlorate resistant mutants "2", "6" and "0/34". Cells were harvested 2.5 h after the transfer to medium with the indicated nitrogen source.

Nitrogen source	*Anabaena* PCC7120	Mutant "2"	Mutant "6"	Mutant "0/34"
Nitrate	47.1	1.1	12.0	0.1
Ammonium	10.0	1.1	2.6	0.2
Arginine	49.4	0.1	14.6	0.1
No N-source	48.9	0.4	10.2	0.2

Activities are determined in µmol nitrite (formed) × mg chlorophyll^{-1} × h^{-1}.

thereby to an increase in arginine concentration. This arginine seems to induce the transcription of a gene coding for nitrate reductase or the molybdenum cofactor. The same mechanism might explain the induction of nitrate reductase activity in *Anabaena* PCC7120. Simon [11] has shown, that after the transfer of cells to nitrate containing medium a mobilization of cyanophycin occurs. This leads to an accumulation of arginine, inducing in turn the synthesis of nitrate reductase. After the transfer to an arginine containing medium this amino acid is taken up leading to an increase of arginine concentration in the cells, which in turn leads to induction of nitrate reductase. However, in contrast to the situation with nitrate as nitrogen source, this increase in nitrate reductase activity does not lead to ammonium production.

Therefore, under these conditions synthesis of nitrogenase in the heterocysts seems to occur and ammonium is produced by nitrogen fixation. Stricker (unpublished [12]) has shown that nitrogen fixation occurs two hours after the transfer of the cells to a medium without combined nitrogen. When nitrogenase is synthesized, induction of nitrate reductase is inhibited. This may be caused by competition of nitrate reductase and nitrogenase for the molybdenum cofactor.

Studies with *Anabaena* PCC7120 and three chlorate resistent mutants have shown that nitrate, arginine and the condition with no combined nitrogen in the medium exert the same effect on nitrate reductase induction. This suggests that in this heterocyst-forming filamentous cyanobacterium the same mechanism for induction of nitrate reductase occurs as that described for *Oscillatoria chalybea*.

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[1] J. Bednarz and G. H. Schmid, Z. Naturforsch. **46c,** 591 (1991).
[2] M. G. Guerrero, J. M. Vega, and M. Losada, Ann. Rev. Plant Physiol. **32,** 169 (1981).
[3] A. Herrero, E. Flores, and M. G. Guerrero, J. Bacteriol. **145,** 175 (1981).
[4] S. N. Bagchi, A. N. Rai, and H. N. Singh, Biochim. Biophys. Acta **838,** 370 (1985).
[5] Y. J. Avissar, Physiol. Plant. **63,** 105 (1985).
[6] J. Bednarz, S. Höper, M. Bockstette, K. P. Bader, and G. H. Schmid, Z. Naturforsch. **44c,** 946 (1989).
[7] L. M. Prescott and M. E. Jones, Analyt. Biochem. **32,** 408 (1969).
[8] S. Ratner, in: Methods in Enzymology (C. P. Colowick and N. O. Kaplan, eds.), **Vol. V,** p. 843, Academic Press, New York 1962.
[9] M. Ohmori, Arch. Hydrobiol. **83,** 485 (1978).
[10] E. Flores, J. M. Romero, M. G. Guerrero, and M. Losada, Biochim. Biophys. Acta **725,** 529 (1983).
[11] R. D. Simon, in: The Cyanobacteria (P. Fay and C. Van Baalen, eds.), p. 199, Elsevier, Amsterdam 1987.
[12] O. Stricker, Diplomarbeit University of Bielefeld, Faculty of Biology (1990).