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Regulation of nitrogen-fixation by different nitrogen sources in the marine non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB1067

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Abstract. The effect of various nitrogen sources on the synthesis and activity of nitrogenase was studied in the marine, non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB1067 grown under defined culture conditions. Cells grown with N_2 as the sole inorganic nitrogen source showed light-dependent nitrogenase activity (acetylene reduction). Nitrogenase activity in cells grown on N_2 was not suppressed after 7 h incubation with 2 mM $NaNO_3$ or 0.02 mM NH_4Cl. However, after 3 h of exposure to 0.5 mM of urea, nitrogenase was inactivated. Cells grown in medium containing 2 mM $NaNO_3$, 0.5 mM urea or 0.02 mM NH_4Cl completely lacked the ability to reduce acetylene. Western immunoblots tested with polyclonal antisera against the Fe-protein and the Mo-Fe protein, revealed the following: (1) both the Fe-protein and the Mo-Fe protein were synthesized in cells grown with N_2 as well as in cells grown with $NaNO_3$ or low concentration of NH_4Cl; (2) two bands (apparent molecular mass of 38000 and 40000) which cross-reacted with the antiserum to the Fe-protein, were found in nitrogen-fixing cells; (3) only one protein band, corresponding to the high molecular mass form of the Fe-protein, was found in cells grown with $NaNO_3$ or low concentration of NH_4Cl; (4) neither the Fe-protein nor the Mo-Fe protein was found in cells grown with urea; (5) the apparent molecular mass of the Fe-protein of *Trichodesmium* sp. NIBB1067 was about 5000 dalton higher than that of the heterocystous cyanobacterium, *Anabaena cylindrica* IAM-M1.

Key words: Cyanobacterium – *Trichodesmium* – Nitrogen-fixation – Nitrogenase

Trichodesmium spp. are non-heterocystous cyanobacteria found in tropical and subtropical seas which are important in mediating a flux of reduced nitrogen from the atmosphere to the ocean. Although *Trichodesmium* does not form heterocysts, the organism is capable of fixing N_2 in the light. It is well documented that nitrogenase activity in heterocystous cyanobacteria is regulated by the presence of combined nitrogen compounds in the culture medium. However, determining whether exogenous combined nitrogen regulates the synthesis and activity of nitrogenase in *Trichodesmium* has been confounded by the difficulties of culturing the organism under controlled laboratory conditions. We have successfully isolated and maintained a clone of *Trichodesmium* in batch culture (*Trichodesmium* sp. NIBB1067), under completely defined conditions (Ohki et al. 1986). The organism, obtained from the Kuroshio current off the coast of Japan, fixes nitrogen when grown with N_2 as the sole inorganic nitrogen source (Ohki and Fujita 1988). Here we report on the effects of three combined nitrogen sources, NH_4^+, NO_3^-, and urea, on the synthesis and activity of nitrogenase in *Trichodesmium* sp. NIBB1067.

Nitrogenase is composed of two proteins, an Fe-protein and a Mo-Fe protein (cf. Haselkorn 1986). The Fe-protein consists of two identical subunits (molecular mass of 25000 to 35000), with a molecular mass of 65000 for the holoprotein. The Mo-Fe protein has a molecular mass of 220000 and is composed of two large subunits (60000 to 65000) and two small subunits (55000 to 60000). Using the acetylene reduction method to assay nitrogenase activity, and immunoblotting to assay protein levels, we examined the regulation of nitrogenase by exogenous combined nitrogen in *Trichodesmium* sp. NIBB1067. Our results reveal that the nitrogen-fixing system of this alga is regulated at two levels: (1) the synthesis of enzyme is regulated at a transcriptional or post-transcriptional level by the presence of urea, and (2) the activity of the Fe-protein is correlated with a shift in electrophoretic mobility, which is believed to be a post-translational modification (Zumft 1985; Smith et al. 1987; Reich and Böger 1989; Ernst et al. 1990a, b).

Materials and methods

Trichodesmium sp. NIBB1067 (Ohki et al. 1986; Ohki and Fujita 1988) was cultured in a modified AQUIL medium of Morel et al.

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(1979) with and without combined nitrogen. The nitrogen compounds and the concentrations used were as follows: $NaNO_3$ (2 mM), NH_4Cl (0.02 mM) or urea (0.5 mM). Cells were grown in the presence of the nitrogen source at least for 15 generations before use. Cultures were maintained at 25°C, under fluorescent light (10 W/m^2) with a 10 h light/14 h dark cycle. Only cells in the exponential or the early linear growth phase were used. *Anabaena cylindrica* IAM-M1 was grown in MDM Medium (Watanabe 1960) without KNO_3 under continuous illumination with incandescent light (6 W/m^2) at 25°C.

Nitrogenase activity was measured by the acetylene reduction technique as previously described (Ohki and Fujita 1988).

For the Western immunoblots of nitrogenase, *Trichodesmium* filaments were collected on a glass fiber filter, frozen and stored in liquid nitrogen. Frozen cells were suspended in 8% SDS containing Na_2CO_3 (0.1 M), dithiothreitol (0.1 M) and phenylmethylsulfonyl fluoride (PMFS) (200 μM). Following sonication and heat denaturation at 80° C for 2 min, the solubilized fraction was collected by centrifugation and used for SDS-PAGE. Proteins were separated by SDS-PAGE on a 15% polyacrylamide gel (Laemmli 1970), and electrophoretically transferred to nitrocellulose. The nitrocellulose was incubated overnight in phosphate buffer (0.01 M, pH 7.2) containing 1% Triton X-100 and 10% dried milk (buffer A). The blots were then separately incubated with antisera raised against the Fe-protein or the Mo-Fe protein from *Rhodospirillum rubrum* (gift of Dr. Paul Ludden, Univ. Wisconsin, diluted 1:1000 with buffer A) for 5 h. The reaction was visualized with a mixture of 1,4-chloronaphthol and H_2O_2 following incubation of the blot with peroxidase conjugated protein A (Bio Rad, 1:1000 dilution).

Chlorophyll *a* was extracted from cells with methanol and determined spectrophotometrically by using the absorption coefficient of Mackinney (1941).

Results and discussion

We first examined the short-term effect of combined nitrogen sources on nitrogenase activity (acetylene reduction) in *Trichodesmium* sp. NIBB1067. Cells grown in nitrogen-free medium were collected and resuspended in fresh medium. Acetylene reduction was measured under illumination since nitrogenase activity in *Trichodesmium* sp. NIBB1067 is light-dependent (Ohki and Fujita 1988). After 1 h, NO_3^-, NH_4^+ or urea was added (Fig. 1A). The final concentration of each combined nitrogen source was 2 mM NO_3^-, 0.02 mM NH_4^+, and 0.5 mM urea. Concentrations of NH_4^+ higher than 0.02 mM, or urea higher than 0.5 mM strongly inhibited growth. Neither NO_3^- nor NH_4^+ inhibited acetylene reduction even after 7 h of incubation while urea inhibited nitrogenase activity after 3 h.

We also examined the effect of long-term growth with NO_3^-, NH_4^+ or urea on nitrogenase synthesis and activity. Cells grown with NO_3^-, NH_4^+ or urea for at least 15 generations were collected, washed and resuspended in medium without combined nitrogen. Cells grown in nitrogen-free medium were used as a control. Cells grown in nitrogen-free medium had high acetylene reduction activity in the light (Fig. 1B). However, cells grown with NO_3^-, NH_4^+ or urea did not show any detectable nitrogenase activity, even after 5 h incubation in nitrogen-free medium.

Cells grown under nitrogen-fixing conditions in nitrogen-free medium synthesized the Mo-Fe protein (Fig. 2A). The apparent molecular mass of the Mo-Fe protein subunit cross-reacted with the antiserum was about 60000. The Mo-Fe protein was also present in cells grown in medium containing 2 mM NO_3^- or 0.02 mM NH_4^+. The Mo-Fe protein was not present in cells grown with 0.5 mM urea.

A Western blot probed with the antiserum raised against the Fe-protein is shown in Fig. 2B. *Trichodesmium* sp. NIBB1067 synthesized this protein when grown in medium containing NO_3^- or NH_4^+ as well as when grown in nitrogen-free medium. Cells grown with urea did not appear to synthesize the Fe-protein. Cells grown with N_2 as the sole nitrogen source and possessing high nitrogenase activity, had two bands (molecular mass of 40000 and 38000) which cross-reacted with the Fe-protein antiserum. Cells grown with NO_3^- or NH_4^+, and lacking nitrogenase activity, contained only one protein band, which corresponded to the high molecular mass

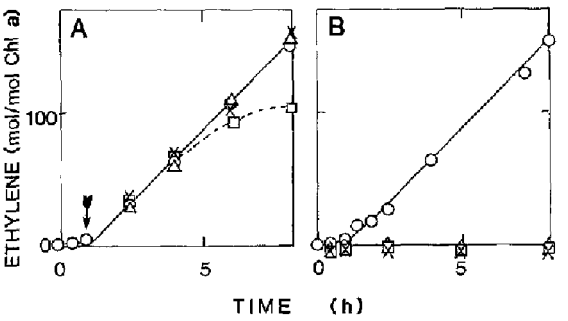

Fig. 1. Short-term (A) and long-term (B) effect of combined nitrogen on aerobic acetylene reduction of *Trichodesmium* sp. NIBB1067. **A** Cells grown in nitrogen-free medium were collected and resuspended in fresh nitrogen-free medium. $NaNO_3$ (2 mM *crosses*), NH_4Cl (0.2 mM, *triangles*) or urea (0.5 mM, *squares*) was added at the time indicated by an *arrow*. No addition of combined nitrogen (*open circles*). **B** Cells grown in medium containing different nitrogen sources were washed and resuspended in nitrogen-free medium. Cells grown without combined nitrogen (*open circles*), with $NaNO_3$ (2 mM, *crosses*), NH_4Cl (0.02 mM, *triangles*) or urea (0.5 mM, *squares*). The gas phase of the reaction vessel was air containing 20% (v/v) of acetylene. Reaction vessels were illuminated by fluorescence light at 10 W/m^2, and the temperature was 25° C. For experimental details, see the text

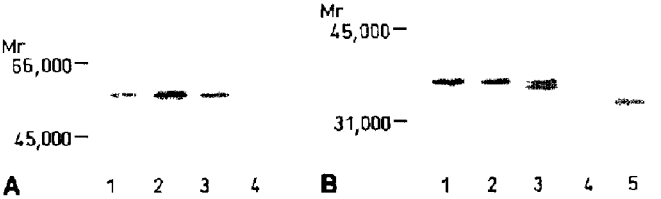

Fig. 2A, B. Western immunoblot analysis of cell extracts of *Trichodesmium* sp. NIBB1067 grown with different nitrogen sources. The samples were loaded with the extracts corresponding to 2.5×10^5 cells/lane. **A** The antiserum raised against the Mo-Fe protein of nitrogenase was used as a probe. Cells grown in nitrogen-free medium (*lane 1*), with $NaNO_3$ (2 mM, *lane 2*), NH_4Cl (0.02 mM, *lane 3*) or urea (0.5 mM, *lane 4*). **B** The antiserum raised against the Fe-protein of nitrogenase was used as a probe. Cells grown in medium with $NaNO_3$ (2 mM, *lane 1*), NH_4Cl (0.02 mM, *lane 2*), without combined nitrogen (*lane 3*) or urea (0.5 mM, *lane 4*). The cell extract of *Anabaena cylindrica* IAM-M1 grown in nitrogen-free medium was applied in *lane 5*. For experimental details, see the text

form present in nitrogen-fixing cells. The apparent molecular mass of the Fe-protein in *Trichodesmium* sp. NIBB1067 was about 5000 dalton higher than that of *Anabaena cylindrica* IAM-M1 (Fig. 2B), but similar in size to the Fe-protein of *Oscillatoria limosa* (Stal and Bergman 1990).

Our results indicate that the nitrogen-fixing system of *Trichodesmium* NIBB1067 is regulated by at least two different ways by added nitrogen sources. Urea suppressed the synthesis of both the Fe-protein and the Mo-Fe protein of nitrogenase. The good correlation between abundance of the Fe-protein and the Mo-Fe protein (Fig. 2A vs. B) suggests that in *Trichodesmium* expression of *nif* genes is highyl coordinated. This is not surprising as the Fe-protein and the Mo-Fe protein are encoded by the *nif HDK* operon and read as a polycistronic message in *Anabaena* sp. PCC7120 (Haselkorn 1986). However without measuring mRNA levels or stability, we cannot distinguish between transcriptional or post-transcriptional control of the *nif* genes by urea.

NO_3^- or low concentration of NH_4^+ did not suppress nitrogenase synthesis. The concentration of NH_4^+ was much lower than that of urea. We could not use NH_4^+ higher than 0.02 mM because of its strong toxicity to *Trichodesmium* growth, but the concentration of NH_4^+ used here may not be high enough to result in suppression of nitrogenase synthesis. The nitrogenase synthesized in cells grown with NO_3^- or NH_4^+ did not show any activity. If the same metabolite regulates both synthesis and activity of the enzyme, the concentration required for deactivation must be lower than that required for suppression of enzyme synthesis.

In cells grown with NO_3^- or NH_4^+, only one band of molecular mass of 40000 cross-reacted with the Fe-protein antiserum. In nitrogen-fixing cells, an additional band of molecular mass of 38000 cross-reacted with the Fe-protein antiserum. Both bands were always found in cells which were actively fixing N_2. Furthermore, the 38000 dalton protein band disappeared when cells were incubated in the dark, which is consistent with the activation of the enzyme by light (Ohki et al. 1991). The correlation between nitrogenase activity and occurrence of the 38 000 dalton protein band indicates that the additional band is not a degradation product but is the active form of the enzyme. In some nitrogen-fixing bacteria, nitrogenase activity is regulated by the reversible modification of the Fe-protein (Zumft 1985), and this modification leads to a 1500 to 5000 dalton increase in apparent molecular mass of the Fe-protein. The modification in *Rhodospirillum rubrum* has been shown to be due to a covalent bonding of ADP-ribose to the protein (Zumft 1985). A change of apparent molecular mass of the Fe-protein accompanied with a change in activity was also observed in cyanobacteria (Smith et al. 1987; Reich and Böger 1989; Ernst et al. 1990a, b) although the ADP-ribosylation has not yet been demonstrated. Our results suggest that the 38000 dalton form is the active form, and indicates that nitrogenase activity in *Trichodesmium* is regulated by what appears to be a modification of the Fe-protein, as in other cyanobacteria.

In conclusion, we have shown that nitrogenase activity of *Trichodesmium* sp. NIBB1067 is not regulated solely by the presence or absence of the Fe-protein of nitrogenase. Enzyme synthesis can be suppressed by a combined nitrogen source, although synthesis is not suppressed by NO_3^- or low concentration of NH_4^+. Furthermore, the Fe-protein appears to undergo a post-translational modification, which leads to inactivation, when cells are grown with NO_3^- or low concentration of NH_4^+.

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