Regulation of nitrogenase activity in relation to the light–dark regime in the filamentous non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB 1067

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A periodicity in nitrogen fixation potential with respect to the light–dark regime was studied in the filamentous non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB 1067. During a 12 h light/12 h dark cycle, potential nitrogenase activity measured by acetylene reduction in the light was insignificant in the dark period, but developed after illumination for 1 to 3 h. Maximum nitrogenase activity was found at the middle of the light period, and activity decreased near the end of the light period. Manipulation of the length of the light and dark periods, and use of the glutamine synthetase inhibitor L-methionine sulfoximine, led to the conclusion that (1) the periodicity in activity was not attributable to an endogenous rhythm, (2) development and maintenance of nitrogenase activity in *Trichodesmium* was regulated by the light period, and (3) the decrease in activity at the end of the light period was due to the accumulation of an intermediate(s) in nitrogen metabolism. The nitrogenase Fe- and MoFe-proteins were always present despite the changes in nitrogenase activity associated with the light–dark cycle. However, a change in apparent molecular mass of the Fe-protein on SDS-PAGE correlated with the change in nitrogenase activity. The results indicate that changes of nitrogenase activity in *Trichodesmium* under a light–dark regime can be attributed to activation and deactivation of the Fe-protein, and that the activation of the protein depends on light.

**Introduction**

Many cyanobacteria can fix molecular nitrogen under autotrophic conditions (Rippka & Waterbury, 1977; Van Baalen, 1987). Since cyanobacteria use oxygenic photosynthesis for autotrophic growth, the resulting aerobic intracellular environment presents a physiological problem for the maintenance of nitrogenase activity. Nitrogenase is rapidly deactivated by oxygen, and cyanobacteria use at least two strategies to protect their nitrogenase system from $O_2$ evolved from photosynthesis. One strategy is a spatial separation of nitrogenase from photosynthetic oxygen evolution by differentiation of specialized cells known as heterocysts. Heterocysts lack photosystem II function, which evolves $O_2$, but are active in nitrogen fixation (Fay, 1973), and are the sites where nitrogenase is localized (Bergman et al., 1986). This differentiation occurs in the organisms of generic groups IV and V of Rippka et al. (1979). The other strategy is a temporal separation of nitrogen fixation from photosynthesis, which has been described in unicellular (Huang et al., 1990; Mitsui et al., 1986; Mullineaux et al., 1981) and filamentous non-heterocystous species (Stal & Krumbein, 1985). In such species, with the exception of *Trichodesmium*, nitrogen fixation oscillates during the day–night cycle, primarily occurring in the dark period.

*Trichodesmium* is a non-heterocystous marine cyanobacterium which can fix nitrogen. Nitrogen fixing activity does not appear to be spatially or temporally separated from photosynthesis in *Trichodesmium*. Immunolocalization studies have shown that nitrogenase is present in most, if not all, of the cells within the...
trichomes of natural populations (Bergman & Carpenter, 1991; Paerl et al., 1989) and of Trichodesmium sp. NIBB 1067 (K. Ohki, P. Falkowski & Y. Fujita, unpublished data). Nitrogen fixation in Trichodesmium has been observed only during the day in natural populations (Capone et al., 1990; Saino & Hattori, 1978), is strictly light dependent, and ceases when photosynthetic activity is inhibited with DCMU (Ohki & Fujita, 1988).

Trichodesmium sp. NIBB 1067, which was isolated from the Kuroshio waters and grown in a defined artificial medium (Ohki et al., 1986), fixes molecular nitrogen in the light when it is grown in a medium free of combined nitrogen (Ohki et al., 1991b). The nitrogenase activity of Trichodesmium sp. NIBB 1067 is highest in cells in the exponential growth phase and low in the stationary growth phase (Ohki & Fujita, 1988). However, when this strain is grown under a light-dark regime, the activity in cells even in the exponential growth phase oscillates diurnally, as has been observed in natural populations. We report here that the oscillation is not circadian in nature, but is due to the dark-deactivation and light-activation of the nitrogenase enzyme.

**Methods**

Trichodesmium sp. NIBB 1067 is the same strain as used in previous studies (Ohki et al., 1991a, 1992). Cells were grown in AQUIL medium (Morel et al., 1979), modified as described by Ohki et al. (1986), without a source of fixed nitrogen, under fluorescent light (daylight type, 40 μmol m⁻² s⁻¹) on a 12 h light/12 h dark regime at 26 °C. All experiments were done with cultures in the exponential growth phase, after more than five cycles of the light-dark regime. The light intensity was 40 μmol m⁻² s⁻¹, and the temperature, 26 °C. Cell concentration at time zero (Figs 1 and 3) was equivalent to approx. 5 × 10⁻¹¹ mol Chl a ml⁻¹. At intervals, cells were collected on nylon mesh (Nytal, Swiss Nylon Plankton Net Cloth, HD20, 20 μm mesh size), immediately resuspended in fresh culture medium free of combined nitrogen, and nitrogenase activity assayed by acetylene reduction. The concentration of the cell suspension for measurement of nitrogenase activity was 3–5 × 10⁻¹⁰ mol Chl a ml⁻¹, which corresponded to 2.5–4.2 × 10⁶ cells ml⁻¹. The acetylene reduction assay was performed as previously described (Ohki & Fujita, 1988). The activity was calculated from the rate of ethylene production during 15 and 30 min incubations in the light (fluorescent light, 40 μmol m⁻² s⁻¹). L-Methionine sulfoximine (MSX; Sigma) was freshly dissolved in H₂O before use. To examine the effect of acetylene on nitrogenase activity, vessels were capped with silicon stoppers and 20% (v/v of total gas phase) acetylene was added after an equal volume of air had been removed with a syringe.

Soluble proteins were separated by SDS-PAGE, transferred to nitrocellulose, and nitrogenase proteins were identified by immunoblotting (Western blotting) as reported previously (Ohki et al., 1991b). Antisera raised against the Fe- or the MoFe-protein of nitrogenase from Rhodospirillum rubrum (generously provided by Dr Paul Ludden, University of Wisconsin, USA) were used as probes. Although the latter antiserum was raised against mixtures of α and β subunits of the MoFe-protein of R. rubrum (P. Ludden, personal communication), it reacted only with the 60 kDa species of the MoFe-protein in Trichodesmium.

Chl a concentration was measured spectrophotometrically in methanol extracts using the absorption coefficient of Mackinney (1941).

**Results**

**Nitrogenase activity in cells grown under light–dark regimes**

Acetylene reduction activity was not detected in the dark period even though the assay was done in the light (Fig. 1). In contrast, the activity increased in the light period immediately preceding the light period was 18 h instead of 12 h.

Fig. 1. Oscillation of the activity of light-dependent acetylene reduction in cells of Trichodesmium sp. NIBB 1067 grown under light–dark regimes. Cells were grown under 12 h light/12 h dark cycles; the last dark period started 12 h before time zero. (a) Activity in cells grown under 12 h light/12 h dark cycles. (b) Same as (a), but the last light period immediately preceding the light period was 18 h instead of 12 h. (c) Same as (a), but the last light period was shortened to 6 h. For determination of acetylene-reduction activity, cells were collected at the times indicated, suspended in fresh nitrogen-free medium and placed in a glass serum bottle, to which acetylene was added to 20% (v/v). Data are expressed as means of duplicate incubations. For experimental details, see the text. Western immunoblot analyses of samples taken from these cultures are shown in Fig. 4 for (b) and in Fig. 5 for (a) and (c).
Regulation of nitrogenase activity in Trichodesmium

Fig. 2. Effect of a short dark pulse on the activity of light-dependent acetylene reduction. Cells were collected at the middle of the light period and suspended in the incubation bottles for the acetylene reduction assay. Bottles were first placed in the light for 30 min, then in the dark for 30 min (●) or 1 h (▲). Following the dark incubations the bottles were illuminated as for the control. The black arrow indicates the beginning of dark incubation; the white arrows show the endpoints. ○, Control without dark pulses. Data are expressed as means of triplicate incubations. Conditions for acetylene reduction were the same as in Fig. 1. The inset shows the time course of the recovery of acetylene reduction activity after a short dark pulse for a longer time range.

period, reaching a maximum at about the middle of the light period and decreasing gradually to zero at the beginning of the dark period (Fig. 1a). Extension of the dark period by 6 h did not alter this pattern, but it did delay the development of the activity by 6 h (Fig. 1b).

Shortening the light period changed the pattern of the active phase substantially. When the light was turned off at the middle of the light period in a 12 h light/12 h dark cycle, the activity decreased abruptly and became undetectable within 1 h (Fig. 1c). Fig. 2 shows the effect of a short dark pulse on nitrogenase activity during the course of acetylene reduction measurement. Nitrogenase activity maintained at the maximum level by the light decreased to about 30% of the control in cells which had been exposed to dark conditions for 30 min, whereas incubation in the dark for 1 h resulted in complete deactivation. Recovery from the dark-deactivation required illumination for at least 1 h.

Nitrogenase activity was not regulated by light alone since it decreased throughout the latter half of the light period and was undetectable prior to the start of the dark period (Fig. 1a, b). However, the period of nitrogenase activity could be extended by addition of the glutamine synthetase inhibitor MSX early in the light period (Fig. 3a). The effect was less pronounced when MSX was added later in the light period. Incubation with acetylene, which competes with N₂ in the nitrogenase reaction, had similar effects to MSX, increasing the duration of acetylene reduction activity following the beginning of the light period (Table 1).

Table 1. Effect of acetylene on decrease in nitrogenase activity during the latter half of the light period

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>Time after light-on (h): 6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>33.0 ± 0.21</td>
<td>32.2 ± 0.25</td>
<td>30.5 ± 0.21</td>
</tr>
<tr>
<td>Air + acetylene*</td>
<td>–</td>
<td>30.3 ± 0.15</td>
<td>24.1 ± 0.15</td>
</tr>
</tbody>
</table>

* The gas phase of the culture vessels was partly replaced by acetylene (approx. 20%, v/v) 6 h after the start of the light period.

Fig. 3. Effect of MSX on light-dependent activity of acetylene reduction (a) and molecular state of the Fe-protein of nitrogenase (b) in cells grown under a light-dark regime. (a) MSX (10⁻⁶ m) was added after illumination for 1 h (●), 3 h (△), 5 h (▲) and 8 h (●). ○, Control without addition of MSX. Data are expressed as means of duplicate incubations. (b) Western immunoblot analysis of the Fe-protein in cells collected at the end of the light period. Antiserum raised against the Fe-protein was used as a probe. The letter for each lane corresponds to the sample labelled with the same letter in (a).
Western immunoblot analysis of extracts from cells collected at different times of the light-dark regime. Cells were grown under 12 h light/12 h dark cycles, but the dark period immediately preceding the light period was prolonged to 18 h. Antisera raised against the Fe- and MoFe-proteins were used as a probe. The number for each lane indicates the time (h) after light-on.

Fig. 4. Western immunoblot analysis of the Fe-protein in cells collected at different times of the light-dark regime. Antiserum raised against the Fe-protein was used as a probe. (a) Cells grown under 12 h light/12 h dark cycles. The number for each lane indicates the time (h) after light-on. (b) Cells grown under 12 h light/12 h dark cycles, but the light was turned off in the middle of the last light period. The number for each lane indicates the time (h) after light-off.

Abundance and molecular form of nitrogenase proteins during the light–dark regime

Western immunoblot analysis of the Fe- and the MoFe-protein of nitrogenase revealed that both proteins were present even when cells were incubated in the dark for 18 h (Fig. 4). However, the Fe-protein increased in apparent molecular mass, as determined by SDS-PAGE, in cells collected at the end of the dark period (Figs 4 and 5a). The Fe-protein changed to a lower molecular mass form within 3 h of the beginning of the light period. Most of the Fe-protein was in this form in the middle of the light period when nitrogenase activity was maximum. The high molecular mass form appeared again in the latter part of the light period and increased toward the end of the light period. When the light was turned off in the middle of the light period, the Fe-protein again increased in apparent molecular mass (Fig. 5b). Thus, changes in nitrogenase activity corresponded with the modification of the Fe-protein.

The effect of MSX on the Fe-protein was to increase the relative abundance of the low molecular mass form at the end of the light period (Fig. 3b). Again, high nitrogenase activity correlated with the presence of the low molecular mass form of the Fe-protein.

Discussion

Oscillation of nitrogenase activity in cells grown under a light–dark regime has been observed for heterocystous (Ernst et al., 1990) and non-heterocystous (Huang et al., 1990; Mitsui et al., 1986; Mullineaux et al., 1981; Stal & Krumbein, 1985) cyanobacteria. In non-heterocystous species, nitrogenase activity is high in the dark and becomes low or insignificant in the light. Interestingly, the oscillation pattern remains unaltered even when the ratio of the light to dark period is changed (Huang et al., 1990; Mitsui et al., 1986; Stal & Krumbein, 1985). Nitrogenase activity of Trichodesmium sp. NIBB 1067 also oscillates over a light–dark cycle, but with high rates of nitrogenase activity only during the light period. Our analyses of the oscillation of nitrogenase activity in Trichodesmium indicate that it is not an endogenous
rhythm, since changes in the light–dark regime immediately cause a shift in the nitrogen fixation periodicity.

The oscillation of acetylene reduction potential in *Trichodesmium* appears to be a result of two factors: the light-dependent activation of nitrogenase and regulation by some aspect of metabolism. A short dark incubation caused a marked and rapid decrease in nitrogenase activity, and reactivation by light occurred after a lag of 30 to 60 min. Since acetylene reduction was measured under illumination, low or insignificant activity after the dark incubation was not due to limitation of energy supply, and/or supply of reductant, from photosynthesis. The enzyme itself must be deactivated during dark incubation, and reactivated by a light-dependent reaction. The occurrence of the lag in reactivation suggests that a component formed in the light is involved in the activating process. MSX (an inhibitor of glutamine synthetase; Stewart & Rowell, 1975) and acetylene (which competitively inhibits nitrogen fixation) allowed higher levels of acetylene reduction to continue in the latter part of the light period. Both of these compounds will reduce the amount of nitrogen available for assimilation, although by two different mechanisms. The decrease in acetylene-reduction activity in the latter part of the light period (Fig. 1) appears to be due to some aspect of the assimilation of nitrogen following several hours of nitrogen fixation. The most plausible explanation is that following several hours of maximum nitrogen fixation rates, the internal pools of some intermediates in nitrogen fixation increase and directly or indirectly result in the decrease in nitrogen fixation rates.

Both the Fe- and the MoFe-proteins of nitrogenase were always present even though the nitrogenase activity changed greatly during the light–dark cycle (compare Fig. 1 and Figs 4 and 5). Although a slight change of the abundance of the two proteins occurred during the light period, the relative abundance of the two proteins changed little. Thus, changes in the activity are primarily due to activation–deactivation of existing enzyme. However, changes in the abundance of the two proteins suggest that turnover of the proteins may occur in parallel with activation–deactivation of the enzyme. A correlation between the nitrogenase activity and the apparent molecular mass of the Fe-protein on SDS-PAGE was noted, as we have previously observed for changes in nitrogenase activity in cells grown with various sources of nitrogen (Ohki et al., 1991b). However, nitrogenase activity did not exactly correlate with the presence of the lower molecular mass form of the Fe-protein. A difference in time between activation–deactivation of the enzyme and modification of the Fe-protein has been observed for *Anabaena variabilis* ATCC 29413 (Reich & Böger, 1989). In *Rhodospirillum rubrum*, nitrogenase is inactivated when one of the two subunits of the Fe-protein has changed to the inactive form (Gotto & Yoch, 1982). If the same occurs in *Trichodesmium*, it could explain both the delay in activation after the appearance of the low molecular mass form of the Fe-protein upon light-on, and the inactivation preceding the loss of the low molecular mass form upon light-off (compare Fig. 1 and Figs 4 and 5).

The regulation of nitrogenase activity in *R. rubrum* has been shown to involve the reversible ADP-ribosylation of the Fe-protein (Ludden & Roberts, 1989). ADP-ribose attached to one of the two subunits of the Fe-protein inactivates the enzyme, and the modified subunit increases the apparent molecular mass of the Fe-protein by about 1.5 kDa as determined by SDS-PAGE (Gotto & Yoch, 1982; Preston & Ludden, 1982). Similar changes in the apparent molecular mass of the Fe-protein have been observed in cyanobacteria, although the occurrence of ADP-ribosylation has not been demonstrated (Ernst et al., 1990; Reich & Böger, 1989; Smith et al., 1987). Since there are some similarities in the correlation of activity with the change in apparent molecular mass of the Fe-protein between *R. rubrum* and *Trichodesmium*, nitrogenase activity in *Trichodesmium* could be regulated by a similar mechanism to that in *R. rubrum*.

Dark-deactivation occurred very rapidly, while light-activation was rather slow, with a lag of 30 to 60 min. These features suggest that, if changes in the activity are simply due to the modification of the Fe-protein, the modification to the inactive form can occur very rapidly, but the reactivation takes much longer. If the modification of the Fe-protein is a result of the activities of activating and deactivating enzymes as in *R. rubrum* (Ludden & Roberts, 1989), the deactivating enzyme in *Trichodesmium* could be constitutive, whereas the activating enzyme must be induced or activated by light. However, we do not know whether the change in molecular mass of the Fe-protein in *Trichodesmium* is due to ribosylation, let alone whether it resembles the activating–deactivating system in *R. rubrum* in other ways.

Although we do not yet understand fully the nature of the modification of the Fe-protein in *Trichodesmium*, it clearly is involved in the regulation of the activity of nitrogenase in this organism over the light–dark cycle (Fig. 4) and in response to the availability of combined nitrogen (Ohki et al., 1991b). A daily cycle of nitrogenase activity in natural populations of *Trichodesmium* has previously been reported (Capone et al., 1990; Saino & Hattori, 1978). In natural populations, a daily cycle of synthesis and degradation has been demonstrated (Capone et al., 1990). In this case, increase in activity in the light (following sunrise) is dependent upon *de novo* synthesis of the Fe-protein. This differs from the
The phenomenon described here for *Trichodesmium* sp. NIBB 1067, even though genetically these organisms must be very closely related (Zehr et al., 1990). However, the natural populations of the Caribbean are in quite a different environment in terms of light quality and quantity, and nutrient availability, to the culture conditions used for *Trichodesmium* sp. NIBB 1067.

The limitation of nitrogen-fixing activity to the light period in *Trichodesmium* would appear to reduce the overall efficiency of the process, since it would be advantageous to continue nitrogen fixation throughout the dark period. Furthermore, nitrogen fixation during the light period poses a physiological problem due to the photosynthetic evolution of oxygen. The lack of nitrogen activity during the dark period may be essential for the assimilation of nitrogenous intermediates produced during the light period, and therefore may increase the overall growth efficiency by conserving energy when sufficient nitrogen has been fixed to supply cell growth over the daily cycle. Most other non-heterocystous cyanobacteria fix nitrogen during the dark period, which is believed to be a strategy for avoiding photosynthetic oxygen evolution (Gallon & Chaplin, 1988; Khamees et al., 1987). Interestingly, *Trichodesmium* appears to have a similar oscillation of nitrogenase activity (although it fixes nitrogen during the light period), but the activity cycle is regulated by light and nitrogen, not by oxygen.

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**References**


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