A Role for Nickel in Cyanobacterial Nitrogen Fixation and Growth via Cyanophycin Metabolism

By ARLENE DADAY, ALISON H. MACKERRAS AND GEOFFREY D. SMITH*

Department of Biochemistry, Faculty of Science, The Australian National University, GPO Box 4, Canberra, ACT 2601, Australia

(Received 1 February 1988; revised 24 May 1988)

When nickel-depleted cells of Anabaena cylindrica with nitrogenase repressed by growth on ammonia or into late stationary phase) were used to re-initiate nitrogen-fixing growth in fresh nickel-depleted medium a pronounced growth lag was observed. The growth lag was not observed in the presence of NiSO₄ (0.68 μM). It was associated with delayed heterocyst differentiation, delayed nitrogenase synthesis and inhibition of pigment synthesis. During the lag a pronounced but transient accumulation of cyanophycin was observed, followed by an accumulation of urea in the growth medium. It is proposed that nickel depletion results in a diversion of ammonia from protein synthesis into cyanophycin, thereby delaying the de novo synthesis of proteins and enzymes required for the synthesis of active nitrogenase.

INTRODUCTION

Nickel ions are known to be essential cofactors for four bacterial enzymes (Thauer, 1983; Hausinger, 1987), of which hydrogenase (Daday & Smith, 1983; Hausinger, 1987) and urease (Berns et al., 1966; Mackerras & Smith, 1986) exist in cyanobacteria. Although the growth of one cyanobacterial species has been reported to be dependent on nickel (Van Baalen & O'Donnell, 1978), the exponential growth rate of nitrogen-fixing Anabaena cylindrica was found to be unaffected, over a range of growth conditions, by omission of nickel from the growth medium (Daday et al., 1985). Thus hydrogenase and urease, and therefore nickel, are apparently not essential for the exponential growth of this organism. However, we here report conditions where the absence of nickel does cause a growth disadvantage to A. cylindrica cells.

METHODS

Cyanobacterial growth in nickel-depleted media. Anabaena cylindrica (ATCC 27899) was grown in a modification of the medium of Allen & Arnon (1955). Stringent precautions were taken to reduce the concentration of nickel ions to negligible levels. All chemicals were Speckure grade (Johnson Matthey), except Na₂EDTA (Analytical Reagent, Univar), KOH, H₂O, K₂HPO₄ and CaCl₂ (each Suprapur grade, Merck). The Na₂EDTA was treated with 0.02% (w/v) dithizone in redistilled chloroform (Thiers, 1957; Klucas et al., 1983) and used to prepare the iron solution from [(NH₄)₂SO₄]FeSO₄. 6H₂O. All components were used at one-eighth the concentration of Allen & Arnon (1955) except the iron solution, which was full strength and Na₂WO₄. 2H₂O, TiO(C₂O₄)₃. yH₂O and Cr₂(SO₄)₃.K₂SO₄. 24H₂O, which were omitted. When nickel ions were required NiSO₄. 6H₂O was added to a concentration (0.68 μM) four times that of the original medium (Daday et al., 1985). All water was from a Millipore reverse-osmosis system with ion-exchange polishing, followed by passage through a Millipore Milli-Q water purification system. All glassware and plastic were washed as described by others (Daday et al., 1985; Thiers, 1957; Klucas et al., 1983) and sparging gases were passed through 5 mm-Na₂EDTA and acid-washed glasswool. Cells grown into late stationary phase in the absence of nickel were used to inoculate culture flasks which were identical in every respect except for the presence of 0.68 μM-NiSO₄. 6H₂O (Speckure) in one of them. When heterocyst-free cells were required NH₄H₂PO₄ (Speckure) was neutralized with KOH and added to the growth medium to give a concentration of 4 mM-NH₄. Cells were grown at 25 °C on a 12 h dark/12 h light cycle at an irradiance of approximately 150 μE s⁻¹ m⁻² (photosynthetically active radiation, PAR). They were sparged with 0.3% CO₂ in air at 170 ml min⁻¹.

0001-4671 © 1988 SGM
The reported measurements were made with cells harvested by centrifugation (8000 g, 10 min) towards the end of the light period. Cell growth was measured as dry weight, determined by centrifuging a sample of cells, washing them once with water and drying them overnight at 85 °C.

**Assays.** Nitrogenase activity was measured by the acetylene reduction technique, as described previously (Lambert & Smith, 1980). Phycocyanin (Craig & Carr, 1968) was determined by measuring the \(A_{620}\) of the supernatant obtained during isolation and measurement of cyanophycin by the method of Simon (1973). Urea was determined in the growth medium after the cells had been removed by centrifugation. The medium was concentrated by rotary evaporation (50 °C) and treated with jack bean urease (Gutmann & Bergmeyer, 1974). Released ammonia was measured with an ammonia electrode (Orion model 95-12). Heterocyst frequencies were determined by cell counts from photomicrographs taken with an Olympus BHA microscope fitted with a phase-contrast attachment (model BH-PC) and camera (PM-10-M).

**RESULTS**

*Growth lag in nickel-depleted A. cylindrica*

Nickel-depleted cells in late stationary phase were used to initiate the reported experiments. The first measurements (Fig. 1) were made 15 d after inoculation. A pronounced lag in the time required to restore exponential nitrogen-fixing growth was observed in the nickel-depleted culture compared with the nickel-sufficient culture (Fig. 1a). The lag was associated with a prolonged yellowing of the cells compared with the control cells, which retained their blue-green appearance throughout. The phycocyanin contents of the cells are shown in Fig. 1(b). Microscopic examination revealed that nickel-deficient cells were devoid of heterocysts and were highly granular in appearance during the lag stage (Fig. 2). Heterocyst frequencies are shown in Fig. 1(c). The growth lag was associated with a delayed synthesis of nitrogenase (Fig. 1d). Eventually nitrogenase appeared in the nickel-depleted cells, after which they reached much the same doubling time (74.3 ± 5.9 h) as nickel-replete cells (66.0 ± 9.6 h). The cells lost their granularity (Fig. 2) and a normal heterocyst frequency developed (Fig. 1c).

The synthesis of cyanophycin [multi-L-arginyl-poly-L-aspartic acid], or cyanophycin granule polypeptide] was closely associated with the growth lag in nickel-depleted cultures. Nickel-depleted cells initially accumulated cyanophycin to a significant proportion of cell dry weight (Fig. 1e). The termination of the growth lag was associated with degradation of this polymer. We also measured urea in the growth medium to determine whether the observations were explicable in terms of the lack of urease in A. cylindrica grown without nickel (Mackerras & Smith, 1986). Urea accumulated in the nickel-deficient growth medium but not in that containing nickel (Fig. 1f), and its appearance was associated with the disappearance of cyanophycin (Fig. 1e). Similar observations were made with cells grown in continuous light and in cultures inoculated with cells in which nitrogenase had been repressed by growth on ammonia.

**DISCUSSION**

This study concerns the effect of nickel on the transition of A. cylindrica from a metabolic state in which heterocyst differentiation and nitrogenase activity are repressed, to one of active nitrogen fixation. Our results suggest that nickel facilitates this transition; although nickel-deficient cells eventually reached growth rates and levels of nitrogen-fixation comparable to nickel-sufficient cells, there was a pronounced delay in their ability to do so, and consequently they underwent a significant growth lag. The transition from one metabolic state to another would require turnover of existing proteins to provide nitrogen for *de novo* protein synthesis (Wood & Haselkorn, 1980), and the marked depletion of phycocyanin (Fig. 1b), which is known to function as a nitrogen reserve, reflects this. The recovery of phycocyanin levels paralleled the induction of nitrogenase activity (Fig. 1b, d), which eventually became the primary source of nitrogen for protein synthesis. Interestingly, the appearance of visually identifiable heterocysts preceded the appearance of active nitrogenase by many hours, even in the control cells. (Fig. 1c, d).
Nickel, cyanophycin and nitrogen fixation

A. cylindrica was grown in nickel-depleted medium to late stationary phase and stored in low light for several months. Such cultures were used to inoculate two 10-litre sets of growth media, one containing 0.68 μM-nickel (■), the other depleted of nickel (□). The first measurements (t = 0) were made 15 d after inoculation. The initial cell concentration (a) was 0.42 μg dry weight (ml culture)^{-1}. Other initial parameters were: phycocyanin (b), 135.8 μg (mg dry weight)^{-1}; nitrogenase activity (d), 0.24 nmol acetylene reduced h^{-1} (mg dry weight)^{-1}; cyanophycin (e), 3.2 μg (mg dry weight)^{-1}; urea content of medium (f), 0.15 mM. Heterocyst frequencies are shown in (c).
The growth lag of nickel-deficient cultures was associated with a large, transient increase in cyanophycin content and we suggest that this is the primary cause of the growth lag and delay in synthesizing active nitrogenase. It appears that nitrogen, which would otherwise be used for de novo protein synthesis, is diverted to net cyanophycin synthesis in nickel-deficient cells. The source of the nitrogen would be turnover of existing proteins such as phycocyanin and also, in our experiments, the 0.18 mM-ammonia which is initially in the growth medium as a consequence of the Specpure iron compound.

The reason why nickel facilitates the transition to nitrogen-fixing conditions is not obvious. The observations are unlikely to be explicable in terms of an absence of hydrogenase activity, which is known to result from an absence of nickel (Daday & Smith, 1983), since in the absence
of nitrogenase activity there would be no source of hydrogen. In terms of known functions for nickel this would therefore suggest that an absence of urease leads to the lag. Urease is apparently required to produce ammonia from arginine, and hence from cyanophycin, via the arginase pathway. The presence of this pathway has been demonstrated in cyanobacteria (Weathers et al., 1978; Gupta & Carr, 1981). However, urea accumulated in the growth medium only towards the end of the lag period, during net cyanophycin degradation (Fig. 1e, f), suggesting that it was a consequence of the growth lag and not its cause. Rather, it appears that the growth lag is due to factors which cause net synthesis of cyanophycin under these conditions, implying a role for nickel in nitrogen metabolism other than as a cofactor for urease and hydrogenase. One possibility is that nickel plays a role in maintaining a rapid turnover of cyanophycin during de novo protein synthesis and an absence of nickel results in a rate of synthesis which exceeds that of breakdown to its constituent amino acids.

The end of the growth lag was coincident with a net breakdown of cyanophycin and accumulation of urea in the medium. *A. cylindrica* lacks urea amidolysae and its urease is absolutely dependent on nickel (Mackerras et al., 1986); hence the urea would remain in the medium and be unavailable for protein synthesis. The concentration of nitrogen in the accumulated urea (23 μM) corresponds closely to 60% of the nitrogen present in the cyanophycin at its peak (60% of 34 μM = 20 μM), implying that three out of the five nitrogens available in an aspartate–arginine pair are released as urea. This may mean that, in addition to the two nitrogens entering urea from arginine via arginase, some of the aspartate nitrogen also finds its way into urea, presumably via the urea cycle. Any nitrogen from cyanophycin which is not trapped as urea would be expected to be available for protein synthesis.

We thank the Australian Research Grants Scheme and the Australian National University Faculties Research Fund for financial assistance. We thank Mrs Nola M. de Chazal, Mr Ronald M. Lynch and Mrs Robyn L. Porter for technical assistance.

**REFERENCES**


