Symbiosis, 6 (1988) 29–36
Balaban Publishers, Philadelphia/Rehovot

Enhancement of Acetylene Reduction *in vivo* by Krebs Cycle Intermediates and by Sugars in the Cyanobacterium *Nostoc muscorum*

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Received March 15, 1988; Accepted June 13, 1988

Abstract

N_2-grown cells of the cyanobacterium *Nostoc muscorum*, provided with Krebs cycle intermediates and sugars, exhibited enhanced acetylene reduction activity, in the light. The enhanced acetylene reduction activity supported by the organic substrates isocitrate, malate, succinate, pyruvate, oxaloacetate, sucrose, fructose, glucose and glucose-6-phosphate, was further enhanced by the addition of low concentration of $NADP^+$. Maximal stimulation was observed with cells preincubated in the light with the organic substrates, under argon. In the dark, the organic substrates enhanced nitrogenase activity to a lower extent. These observations suggest that Krebs cycle metabolites, $NADP^+$ and sugars are taken up by *Nostoc muscorum* cells in the light and in the dark, and are further metabolized to donate reductants to nitrogenase in the heterocysts, and stimulate its activity. The heterotrophic capacity demonstrated by *Nostoc muscorum*, may be significant for this organism under natural growth conditions, and indicates its potential for establishing symbiotic interaction with suitable hosts, including higher plants.

Keywords: *Nostoc muscorum*, Cyanobacteria, Krebs cycle, N_2-fixation

Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid

1. Introduction

One way of providing reductants to nitrogenase in cyanobacteria is the oxidation of organic compounds (Apte et al., 1978). Intermediates of the oxidative pentose phosphate pathway or Krebs cycle may be oxidized by $NADP^+$-dependent dehydrogenases, and generate NADPH. The electrons are further transferred to nitrogenase through ferredoxin, under conditions of high ratios of NADPH to $NADP^+$ in the cell (Bothe, 1970; Tel-Or and Stewart, 1977).

Earlier observations showed enriched activity of the oxidative pentose phosphate pathway enzymes in the heterocyst (Winkenbach and Wolk, 1973) and an incomplete Krebs cycle in cyanobacteria, due to the absence of α-ketoglutarate dehydrogenase (Smith et al., 1967). Therefore, it was assumed that the oxidative pentose phosphate pathway is the major route for the provision of reductant for cyanobacterial nitrogenase (Lex and Carr, 1974). Recently, levels of isocitrate dehydrogenase were shown to be higher than the activity of glucose-6-phosphate dehydrogenase in heterocysts of *Nostoc muscorum* (Karni et al., 1982; Karni and Tel-Or, 1983). A slow rate of incorporation of sugars by cyanobacteria (Smith, 1982) and deactivation of glucose-6-phosphate dehydrogenase in the light by thioredoxin in the vegetative cells (Cossar et al., 1984) were also observed. These facts raise the question of whether the oxidative pentose phosphate pathway or the Krebs cycle is the most efficient route to donate reductants for cyanobacterial N_2-fixation.

Recently, its was found that heterocysts possess all the glycolytic enzymes and part of the Krebs cycle enzymes (Neuer et al., 1983). Hence, sugars entering the heterocysts may be metabolized by glycolysis and by the Krebs cycle complemented with the glyoxylate junction (Fogg et al., 1973). Regeneration of reduced pyridine nucleotides by isocitrate dehydrogenase and malate dehydrogenase is therefore possible in cells provided with sugars and organic acids.

This study presents evidence for enhanced N_2-fixation by *Nostoc muscorum* cells *in vivo* supported by the addition of Krebs cycle intermediates and sugars.

2. Materials and Methods

Organism and growth

Nostoc muscorum, strain 7119, was grown as described before (Karni et al., 1982).

Acetylene reduction assay

Cultures of *N. muscorum* at optical densities of 0.6 O.D. (660 nm) were harvested by centrifugation at 2,500 g for 10 min. The cells were suspended in 25 mM HEPES buffer, pH 7.8, to an optical density of 0.5 O.D., in vials sealed with rubber stoppers under 10% acetylene in air. Illuminated samples (I = 5 W m^{-2}) were incubated on a shaker at 25°C, for 2 hr. Ethylene formation was followed by analysis on a Gow-Mac, Model 69-110 gas chromatograph, provided with Poropack-N column and flame ionization detector. The results were expressed as nmol C_2H_4 h^{-1} mg dwt^{-1}. Cells dry weight was determined after filtration on 0.45 μ filter paper and drying at 70°C for 2 hr.

Effects of sugars and organic acids on acetylene reduction activity

Cells of *N. muscorum* were incubated for 22 hr in light (I = 5W m^{-2}), under argon (to exclude N_2) with 0.5 mM of one of the following substrates: glucose-6-phosphate, glucose, fructose, sucrose, pyruvate, isocitrate, succinate, malate and oxaloacetate, and with $NADP^+$, 0.05 mM. Acetylene (10% V/V) was injected then and the cells were incubated for 2 additional hours in the light, and ethylene formation followed as described.

3. Results and Discussion

Nitrogenase activity of cells provided with isocitrate was two-fold higher than that of the control cells, indicating uptake and metabolism of isocitrate by the cells (Table 1). $NADP^+$, at a low concentration (0.05 mM), further enhanced the nitrogenase stimulated activity in the presence of isocitrate. It is suggested that $NADP^+$ was taken up by the cells, and enhanced the electron transfer to nitrogenase by being substrate for dehydrogenase. Incorporation of $NADP^+$ by *N. muscorum* may be of physiological significance for this organism which has to provide the high demand of reduced pyridine nucleotide for nitrogenase activity (Karni and Tel-Or, 1983).

Sucrose was found to be the most effective substrate among the tested sugars (sucrose > fructose > glucose > glucose-6-phosphate) (Fig. 1). The fact that sucrose-enhanced nitrogenase activity was much higher than the activity enhanced by its hydrolysis products glucose and fructose, may be

Table 1. The effects of isocitrate and of $NADP^+$, on nitrogenase activity of *Nostoc muscorum* cells

Conditions	Acetylene reduction ($nmol\ C_2H_4 \cdot h^{-1} \cdot mg\ dwt^{-1}$)
Light	5.5
+ 0.5 mM isocitrate	12.6
+ 0.5 mM isocitrate + 0.05 mM $NADP^+$	30.9

Cells were incubated in HEPES buffer, 25 mM, pH 7.8, in rubber stoppered bottles (14 ml) in the light (I=5 Wm^{-2}) on a shaker at 25°C for 22 hr. 10% acetylene was then added for 2 hr incubation and ethylene production was determined.

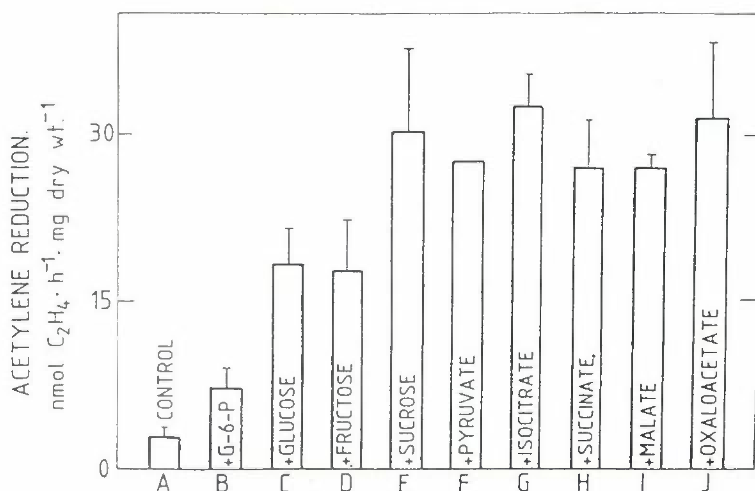

Figure 1. The effects of sugars and organic acids on acetylene reduction activity in intact cells of *Nostoc muscorum*. All the substrates were added in a concentration of 0.5 mM. $NADP^+$, 0.05 mM was added in all treatments.

explained by a faster uptake of sucrose by *N. muscorum* cells. Furthermore, it may also indicate a faster translocation of sucrose than that of glucose or fructose from the vegetative cells to the heterocysts.

The organic acids, pyruvate, isocitrate, succinate, malate and oxaloacetate, were highly active in stimulating nitrogenase activity, suggesting metabolism of the organic acids by enzymes of the incomplete Krebs cycle. The fact

that succinate, malate, and oxaloacetate were as effective as isocitrate and pyruvate in stimulating nitrogenase activity, suggests that the incomplete Krebs cycle indeed functions in the cells of *N. muscorum.* These results also suggest that glyoxylate junction (Fogg et al., 1973) links the two sides of the incomplete Krebs cycle in *N. muscorum.* Hence the Krebs cycle in these cells is active, and its contribution for the N_2-fixation process is important.

The experiments described so far were conducted in two phases: the phase of preincubation under argon for 22 hr in the presence of substrates, and the phase of nitrogenase activity, during further 2 hr incubation under acetylene. The effect of light on the two phases was tested in order to assess the need for light driven ATP synthesis (Fig. 2).

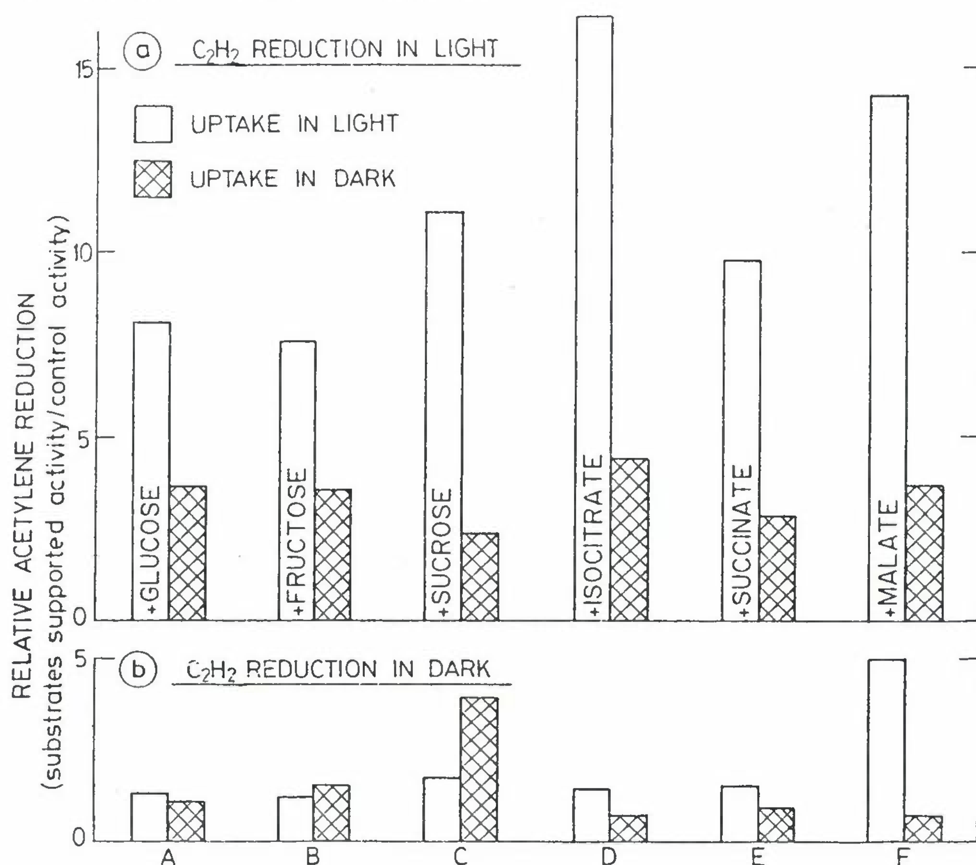

Figure 2. A comparison of the substrate stimulated acetylene reduction activity in the light (a) and in the dark (b). The experiments were conducted as explained in Materials and Methods. The tested substrates were: A — glucose, B — fructose, C — sucrose, D — isocitrate, E — succinate, F — malate.

Rates of C_2H_2-reduction (Fig. 2a) were about three-fold higher when the preincubation was carried out in the light as compared to the dark for each of the substrates tested. These results suggest a major contribution of light-generated energy to uptake and metabolism of sugars and organic acids. When C_2H_2-reduction was assayed in the dark (Fig. 2b), sucrose, taken up in the dark, and malate, taken up in the light, were found to be the most effective substrates supporting nitrogenase activity. The higher effect of sucrose in the dark, was probably involving the reaction of glucose-6-phosphate dehydrogenase in the vegetative cells. This enzyme was shown to be inhibited by light (Cossar et al., 1984), and therefore is fully active only in the dark. Among the organic acids, malate-supported nitrogenase activity, when uptake proceeded in the light, and nitrogenase was assayed in the dark (Fig. 2b), was the most effective.

Thus stimulation of C_2H_2-reduction by organic substrates was dependent on light driven ATP synthesis, both for the uptake of substrates an for nitrogenase activity *per se*. However, the dependence on light driven ATP synthesis was not obligatory. Stimulation of the C_2H_2-reduction was also observed when the uptake of the organic substrates proceeded in the dark.

The results reported here lead to a reevaluation of the function of the incomplete Krebs cycle in the metabolism of the N_2-fixing cyanobacteria. In addition to the proven high isocitrate dehydrogenase activity (Karni et al., 1982; Karni and Tel-Or, 1983), it seems likely that other Krebs cycle enzymes, such as malate dehydrogenase and succinate dehydrogenase, are highly active, possibly due to their induction by the exogeneous substrates malate and succinate.

This study suggests that *N. muscorum* cells possess mechanisms for uptake and metabolism of a large number of substrates, in the light and in the dark. Other cyanobacterial species, e.g. *Aphanocapsa* (Rippka, 1972) and *Nostoc* Mac (Bottomley and Van Baalen, 1978) have active uptake mechanisms for glucose, and *Anabaena azollae* (Tel-Or et al., 1983) for fructose. However, only *N. muscorum*, as shown in this report, is capable of utilizing such a broad range of sugars and organic acids.

This heterotrophic capacity could be of great importance for *N. muscorum* in specific environments. Natural populations of cyanobacteria live in light limiting conditions, e.g. precipitates of water reservoirs, or clustered in large dense colonies. Hence, *N. muscorum* may depend on exogeneous substrates for its metabolism under these conditions.

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