Biochimica et Biophysica Acta 891 (1987) 115–120
Elsevier

BBA 42508

Electron donation to nitrogenase in a cell-free system from heterocysts of *Anabaena variabilis*

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(Received 21 December 1986)

Key words: Heterocyst; Nitrogenase; Thioredoxin; Substrate; Cyanobacterium; (*A. variabilis*)

Glycogen degradation and intermediates of the oxidative pentose-phosphate cycle supported high rates of acetylene reduction in a cell-free heterocyst preparation. This activity could be further increased by addition of ferredoxin and nitrogenase, isolated from heterocysts; rates well over 400 μmol C_2H_4/mg Chl per h were observed. Under these conditions ferredoxin from vegetative cells was inactive. Light and especially dithiothreitol inhibited substrate-dependent nitrogenase activity, pointing to an obligate involvement of glucose-6-phosphate dehydrogenase in carbohydrate degradation as the main electron source for nitrogenase.

Introduction

Considerable progress has been made in elucidating ferredoxin-reducing electron-transport pathways for nitrogen fixation in heterocysts of cyanobacteria [1,2]. Recently, a special plant-type ferredoxin (I) from heterocysts was shown to reconstitute nitrogenase-dependent reactions [3]. With isolated heterocysts [4] and more clearly with freeze-thawed heterocysts [5] or a cell-free system thereof [6], evidence has been obtained that ferredoxin could be photoreduced with electrons from hydrogen or NADH. In the dark, reduced ferredoxin could be generated by pyruvate : ferredoxin oxidoreductase [7], an enzyme present in heterocysts [8]. However, negligible or low rates of pyruvate-dependent nitrogen fixation have been observed so far [4,8,9–12]. Ferredoxin : NADP oxidoreductase has therefore been regarded as the main catalyst for ferredoxin reduction. This reaction, however, required a high NADPH/NADP ratio [6,13,14], whereas the key enzymes supposed to be involved in NADPH-generation, such as glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase, were negatively regulated by NADPH, ATP and light [13,16–21]. Yet, both a high reductant and a high-energy charge generated in the light are prerequisite for high nitrogen-fixing activities [5,6,13–15]. Conflicting results have been reported concerning the light inactivation of glucose-6-phosphate dehydrogenase via thioredoxin in heterocysts [13,21–23]. With a homogenate from heterocysts we observed high rates of acetylene reduction in the dark if the 'in-vitro' system was supplemented with an ATP-generator and substrates of the oxidative pentose phosphate pathway. Light inhibited these activities by approx. 50% [6]. In the present investigation we continued screening for active intermediates supporting nitrogenase activity in heterocyst homogenates and in particular investigated the regulatory role of light and the possible involvement of thioredoxin.

Abbreviation: Chl, chlorophyll.

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Materials and Methods

Culture conditions of *Anabaena variabilis*, Kütz. (ATCC 29413), heterocyst isolation, French-press treatment of heterocysts and measurements of nitrogenase activities in the presence of an ATP-generating system were performed as in Ref. 6. Partially purified nitrogenase from heterocysts was prepared according to Ref. 24.

Heterocyst ferredoxins were isolated from the 350 000 × *g* supernatants of the heterocyst homogenate [3]. The ferredoxin containing fraction was diluted 1 : 4 with cold distilled water and placed on a small DEAE Sepharose CL6B (Pharmacia) column (1.5 × 3 cm), equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was washed with 30 ml buffer, containing 0.1 M NaCl and developed with a linear gradient of NaCl (0.1–0.6 M, total volume 100 ml) at a flow rate of 0.25 ml/min. First, ferredoxin I eluted at approx. 0.32 M NaCl, then ferredoxin II at approx. 0.41 M NaCl. A complete biochemical characterisation of both ferredoxins will be presented elsewhere.

Several different heterocyst homogenate preparations were used in this study and each experiment was repeated at least two times. Chlorophyll was determined after methanol extraction according to Mackinney [25]. Depending on the preparation used, 20–25 mg protein/ml of heterocyst extract corresponded to 1 mg Chl/ml (see also accompanying paper: Ref. 36). Biochemicals and enzymes were purchased from Boehringer (Mannheim, F.R.G.) or Sigma (München, F.R.G.).

Results and Discussion

Substrate-dependent nitrogenase activities

As shown in Table I, glucose-6-phosphate-dependent nitrogenase activities of heterocyst homogenates were mainly limited by the amount of active nitrogenase present in the assay. Completing our previous data on reconstitution of electron transport to nitrogenase [3], we show here that substrate-supported acetylene reduction was further stimulated by ferredoxin I from heterocysts, whereas ferredoxin II from heterocysts and vegetative cell ferredoxin were inactive (Table I, cf. Ref. 3). This is in contrast to observations showing a relatively unspecific reconstitutional activity of

TABLE I

INCREASE OF GLUCOSE-6-PHOSPHATE-DEPENDENT NITROGENASE ACTIVITY BY HETEROCYST FERREDOXIN (I) AND NITROGENASE IN HETEROCYST HOMOGENATES

All assays contained as cofactors: 20 μM NAD/NADP. Concentration of glucose 6-phosphate: 2.5 mM. Ferredoxins I and II were isolated from heterocysts, ferredoxin (vc) from vegetative cells; concentration of ferredoxin I in (B) was 5 μM. Concentration of heterocyst nitrogenase: 66 μg protein/ml.

Electron donor + other additions	Nitrogenase activity (μmol C_2H_4/mg Chl per h)
A. Glucose 6-phosphate	
no addition	27
+ ferredoxin I (0.3 μM)	42
+ ferredoxin I (1 μM)	79
+ ferredoxin I (5 μM)	96
+ ferredoxin I (10 μM)	90
+ ferredoxin II (5 μM)	20
+ ferredoxin vc (5 μM)	23
B. Glucose 6-phosphate + ferredoxin (I)	
no addition	94
+ nitrogenase (5 μl)	187
+ nitrogenase (10 μl)	302
+ nitrogenase (15 μl)	448

nitrogen fixation by cyanobacterial ferredoxins [26,27].

Addition of a partially purified nitrogenase preparation from heterocysts led to a further increase in acetylene reduction and rates well over 400 μmol C_2H_4 formed/mg Chl per h were obtained with glucose 6-phosphate as substrate (Table IB). These rates are compatible with the nitrogen fixation rates of heterocystous cyanobacteria.

Among the substrates supporting nitrogenase activity, not only di- and monosaccharides such as maltose, sucrose, glucose and fructose were active, but glycogen as well (Table IIA). This indicated that heterocysts contained the complement of glycogen degrading enzymes, which together with hexokinase and fructokinase finally led to glucose 6-phosphate. Glycogen, which is accumulated in heterocysts [28], can be used effectively as reductant source for nitrogen fixation in these cells.

As shown in Table IIB, glucose 6-phosphate, 6-phosphogluconate, fructose 6-phosphate and ribose 5-phosphate supported both comparable

TABLE II

NITROGENASE ACTIVITY OF A HETEROCYST HOMOGENATE SUPPORTED BY PHYSIOLOGICAL SUBSTRATES IN THE LIGHT AND IN THE DARK

A and B were different experimental sets with different homogenates; substrate concentrations: 2.5 mM; glycogen: 10 mg/ml; cofactors: 20 μM NAD/NADP, and in (B) additionally 50 μM coenzyme A was added.

Substrate	Nitrogenase activity (μmol C_2H_4/mg Chl per h)		
	dark	light	% inhibition
A			
No addition	1.1	0.5	54.5
Glycogen	18.5	12.0	35
Maltose	14.9	–	–
Sucrose	8.0	–	–
Glucose	27.7	–	–
Fructose	23.1	–	–
B			
Glucose 6-phosphate	29.0	15.3	43.8
6-Phosphogluconate	38.8	26.8	31
Fructose 6-phosphate	27.8	–	–
Fructose 1,6-bisphosphate	23.0	13.1	43
Ribose 5-phosphate	31.0	18.6	40
Dihydroxyacetonephosphate	20.5	11.9	42
Pyruvate/oxaloacetate	17.7	12.9	27
Isocitrate	18.0	10.8	40

and high acetylene reduction activities with NAD(P) added as cofactor(s). Fructose 1,6-bisphosphate and dihydroxyacetone phosphate usually were somewhat less active as substrates in these extracts (60–70%). The rates with (iso)citrate and the donor couple pyruvate/oxaloacetate were approx. 50% and less of the control. Pyruvate or oxaloacetate added separately doubled or tripled the low basal nitrogenase activity of the homogenate (e.g., 0.8 μmol C_2H_4 formed/mg Chl per h; basal activity in this experiment), whereas both pyruvate and oxaloacetate added together in equimolar amounts increased acetylene reduction more than tenfold (10.4 μmol C_2H_4/mg Chl per h). Possibly the removal of inhibiting concentrations of acetylcoenzyme A or other intermediates of the pyruvate-degrading pathway was necessary. A further crucial factor was the optimal concentration of coezyme A, as already noted by others [8,9,11]. This points to a highly regulated pyruvate metabolism, which together with the apparent oxygen sensitivity of pyruvate: ferredoxin oxidoreductase might explain previous results, where pyruvate was inactive as substrate. In addition, the activity of this enzyme might be in general much lower than compared to the dehydrogenases of the oxidative pentose phosphate pathway (cf. Ref. 8).

The following substrates supported negligible nitrogenase activity in the presence of NAD(P) and/or coenzyme A as cofactors, whether assayed in the dark or in the light: 2-keto-3-desoxy-6-phosphogluconate, glycerate, glycerine, hydroxypyruvate, mercaptopyruvate, cysteine, β-alanine, lactate, acetate, acetylcoenzyme A, 2-oxoglutarate, malate and succinate. As will be shown in the following paper [36] 3-phosphoglycerate, phospho*enol* pyruvate and glyoxylate were rather inhibitory.

These findings deserve some comments: the Entner–Doudoroff pathway, which might supply reducing equivalents via pyruvate through 2-keto-3-desoxy-6-phosphogluconate aldolase, seems not to operate in heterocysts as also observed with unicellular cyanobacteria [29]. According to the results of Jüttner [30], alanine is transported from vegetative cells into heterocysts possibly serving as a precursor for reducing equivalents via alanine dehydrogenase [31]; however, alanine neither supported nitrogenase activity in the dark nor in the light. Smith et al. [32] reported glyoxylate stimulated hydrogen formation by *Anabaena cylindrica*, suggestive of electron supply to nitrogenase, an effect, however, requiring long incubation periods (24 h). As indicated above, glyoxylate inhibited nitrogenase activity in the cell-free heterocyst system, in accordance with recent observations of Neuer and Bothe [11].

Effect of light on substrate dependent nitrogenase activities

Inhibition of glucose-6-phosphate dehydrogenase from cyanobacteria by thioredoxin is now well established [19,21,23]. Dithiothreitol inhibition of this key enzyme of hexose dissimilation required the presence of low amounts of thioredoxin [21,23]. As shown previously [6] and now in more detail, light and especially dithiothreitol

acted as very potent inhibitors of dark nitrogenase activity.

Inhibition by light was approx. 40–45% with all active substrates tested including glycogen, but somewhat less pronounced with 6-phosphogluconate and with the pyruvate/oxaloacetate couple as substrates (Table II). Light inhibition of substrate-dependent nitrogenase activity occurred already at very low light intensities (approx. 50 μmol photons/m^2 per s, Fig. 1) probably sufficient to reduce the ferredoxin/thioredoxin system. A similar light saturation curve was obtained with the light-induced switch-off of direct ferredoxin reduction by the NADPH/NADP : ferredoxin oxidoreductase donor system [34]. Regulatory effects are obviously fully exhibited at light intensities far below saturation of photosynthetic electron transport ($\gg$ 300 μmol photons/m^2 per s, compare Fig. 2 in Ref. 6).

These results are at variance with those published by Neuer and Bothe [11]. In a cell-free system derived from heterocysts of *Anabaena variabilis,* they observed a (15–25)-fold stimulation of nitrogenase activity by light with glucose 6-phosphate or pyruvate, respectively, as electron

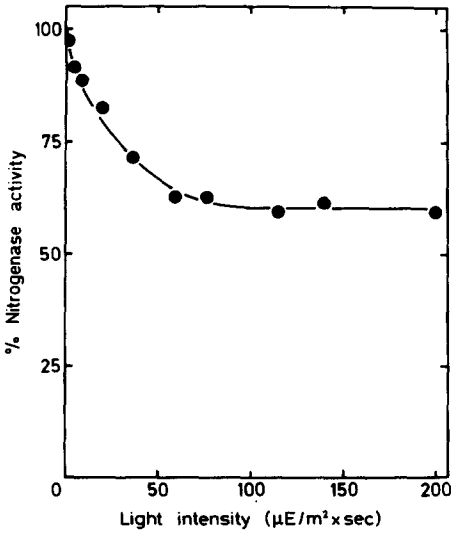

Fig. 1. Inhibition by increasing light intensities of glucose 6-phosphate supported nitrogenase activity in a heterocyst homogenate. The assay contained: 2.5 mM glucose 6-phosphate and 20 μM NAD(P). Light intensity was varied by neutral density filters; 100% activity was 30 μMol C_2H_4/mg Chl per h.

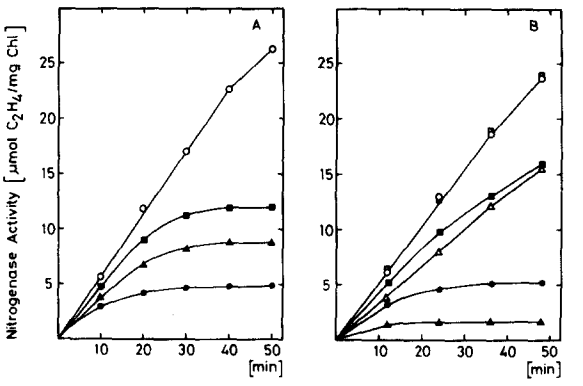

Fig. 2. Influence of dithiothreitol on substrate-dependent nitrogenase activity in heterocyst homogenates. (A) Control with glucose 6-phosphate (○), dithiothreitol added: 0.5 mM (■), 1.0 mM (▲) and 5 mM (●). (B) Activities in the absence and presence of 2 mM dithiothreitol with 6-phosphogluconate (□, ■), ribose 5-phosphate (○, ●) and dihydroxyacetone phosphate (△, ▲) as substrates. Substrate concentrations: 2.5 mM; cofactors: 20 μM NAD/NADP.

donors. It should be noted though that maximum dark activities were very low to begin with (0.2 nmol C_2H_4 per mg protein per min).

As shown in Fig. 2A, addition of dithiothreitol in the dark led to almost complete inhibition of substrate-dependent nitrogenase activities. Inhibition of glucose 6-phosphate supported acetylene reduction increased with time and with increasing concentrations of dithiothreitol, as is expected for a thioredoxin-mediated enzyme modification. This was true for all active substrates listed in Table II, with two exceptions. At a given concentration of dithiothreitol, 6-phosphogluconate-supported nitrogenase activity was much less inhibited, whereas with dihydroxyacetone-phosphate nitrogenase activity was blocked severely and immediately. Since 6-phosphogluconate dehydrogenase is not affected by dithiothreitol (see following paper [36]), initial excess of 6-phosphogluconate could provide nitrogenase with reductant (NADPH) for some time. Metabolisation through the oxidative pentose phosphate cycle eventually would include thioredoxin-sensitive glucose-6-phosphate dehydrogenase and lead to a decrease of electron supply to nitrogenase, as is observed experimentally (Fig. 2B).

We further suggest that dihydroxyacetone phosphate in equilibrium with glyceraldehyde 3-phosphate (derived from the oxidative pentose-

phosphate pathway) forms fructose 1,6-bisphosphate catalyzed by aldolase, which is moderately active in heterocysts (see following paper Ref. 36 and Ref. 8). Fructose 1,6-bisphosphate could be further metabolized to glucose 6-phosphate, which, however, would be generated at low rates only; this might not be sufficient to prevent immediate inactivation of glucose-6-phosphate dehydrogenase by dithiothreitol. As already pointed out [21], only high concentrations of glucose 6-phosphate (together with glutamine) override the deactivating effect of the ferredoxin/thioredoxin system.

From the data presented and those of Udvardy et al. [23], we infer a similar function of thioredoxin, present in vegetative cells and heterocysts, in regulating carbohydrate breakdown via glucose-6-phosphate dehydrogenase by light deactivation/dark activation. Crawford et al. [35] suggested a regulation of the pentose-phosphate pathway by the ferredoxin/thioredoxin system to function only in oxygenic photosynthetic prokaryotes, but not in cells with anoxygenic photosynthesis, such as *Chromatium*. However, heterocysts seemed to have retained the possibility of thioredoxin-mediated enzyme modulation, although an oxygenic Photosystem II is absent. Obviously high oxygen concentrations are not necessary to reverse the activating/inactivating effects of reduced thioredoxin. Alternatively, other enzyme modulators may be of similar importance, such as oxidized glutathione [17]. Dark activation of glucose-6-phosphate dehydrogenase in addition might involve glucose-6-phosphate derived from carbohydrates, supplied either by vegetative cells or by endogenous reserves within the heterocysts, such as glycogen [21].

Acknowledgement

This work was supported by grants from the Deutsche Forschungsgemeinschaft. We wish to thank Marion Oehri for excellent technical assistance, Prof. B. Bowien for a gift of 2-keto-3-desoxy-6-phosphogluconate (Li-salt) and Prof. P. Böger for support.

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