A distinct ferredoxin for nitrogen fixation isolated from heterocysts of the cyanobacterium *Anabaena variabilis*

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Ferredoxin from heterocysts of *Anabaena variabilis* has been isolated and its biological activity compared to ferredoxin obtained from vegetative cells of the same organism. Both ferredoxins catalyze equally effective $NADP^+$ photoreduction by heterocyst thylakoids. When photoreduced, both ferredoxins transfer electrons to nitrogenase from *A. variabilis*, with heterocyst ferredoxin being twice as active as vegetative cell ferredoxin. In the dark, however, only heterocyst ferredoxin is able to link reducing power, generated by soluble systems, such as H_2/hydrogenase (from *Clostridium pasteurianum*) and NADPH/ferredoxin:$NADP^+$ oxidoreductase (from *A. variabilis*), to the cyanobacterial nitrogenase. Using heterocyst homogenates with glucose 6-phosphate as electron donor, only ferredoxin from heterocysts is able to stimulate nitrogenase activity further.

Heterocyst *Nitrogen fixation* *Ferredoxin* *Reconstitution* *Electron transport* Anabaena variabilis

1. INTRODUCTION

The presence of two different molecular species of ferredoxin in one organism has been documented not only for (photosynthetic) bacteria [1–4], but also for higher plants and cyanobacteria (review [5]). Comparative studies on biochemical properties and biological activities of ferredoxins from cyanobacteria yielded the following results: some species contain two chloroplast-type ferredoxins with little difference in spectroscopic properties (optical, EPR), isoelectric point, and molecular mass. However, amino acid composition and redox potentials were different; e.g., ferredoxin I from *Nostoc*, strain MAC, had a midpoint potential of −350 mV, while that of ferredoxin II was reported to be −455 mV (pH 8.0) [6–10].

Comparison of biological activities of both ferredoxins gave contradictory results: Hutber et al. [11] and Hutson et al. [12] found ferredoxin II to be about twice as active as ferredoxin I in $NADP^+$ photoreduction with both higher plant chloroplasts and *Nostoc* photosynthetic membranes. In contrast, Wada et al. [13] reported a lower activity of ferredoxin II compared to ferredoxin I with spinach chloroplasts and no difference between both ferredoxins when *Nostoc* photosynthetic membranes were used. Ferredoxin II from *Nostoc* was also less active in coupling reducing power of illuminated spinach chloroplasts to *Bacillus polymyxa* nitrogenase, whereas ferredoxin I and II isolated from *Aphanothece sacrum* showed similar rates of acetylene reduction [13]. Additionally, Hutber et al. [11] compared ferredoxin I and II from *Nostoc* in supporting pyruvate oxidation by the phosphoroclastic system of *Clostridium pasteurianum* and found almost identical activities. In total these data show that the biological significance of different ferredoxins in cyanobacteria is not yet understood.

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Abbreviations: ATCC, American Type Culture Collection; PS, photosystem

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In filamentous cyanobacteria aerobic nitrogen fixation is restricted to specialized cells called heterocysts, which differentiate from vegetative cells upon exhaustion of nitrogen in the growth medium. A thorough investigation of the properties of ferredoxin from heterocysts has not been performed (cf. [14]). Here we describe the biological activities of ferredoxin isolated from heterocysts as distinct from ferredoxin isolated from vegetative cells of *A. variabilis*. Various native electron transport systems were reconstituted in vitro comprising ferredoxin-dependent $NADP^+$ photoreduction and 3 different electron transport systems requiring ferredoxin as direct electron donor to nitrogenase.

2. MATERIALS AND METHODS

A. variabilis (ATCC 29413) was grown photoautotrophically in nitrogen-free or NO_3^--supplemented (20 mM KNO_3) nutrient solution [15]. Details of heterocyst isolation are also described in [15]. Isolation of heterocyst thylakoids, cytochrome *c*-553, ferredoxin:$NADP^+$ oxidoreductase and ferredoxin (from vegetative cells, grown in the presence of KNO_3), followed the procedures already published [16]. Hydrogenase from *C. pasteurianum* was prepared according to [17].

Heterocyst ferredoxin was obtained from French press-treated heterocysts as a light-brown supernatant after centrifugation of the extract at 350000 × *g* for 5 h (for details see [16]). This supernatant was applied to a DEAE-cellulose column (DE-52, Whatman, 1.5 × 7 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The column was washed with Tris buffer containing 0.25 M NaCl and thereafter ferredoxin was eluted with 0.4 M NaCl. The reddish-brown fractions were diluted 1:5 with cold distilled water and rechromatographed on a small DE-52 column (1 × 4 cm), equilibrated with 50 mM Tris-HCl buffer, pH 8.0. Concentrated ferredoxin was eluted with 0.4 M NaCl. As checked by SDS-polyacrylamide gel electrophoresis, heterocyst ferredoxin was free of other proteins.

Nitrogenase activities were measured in 8-ml vials closed with septum stoppers; acetylene reduction was detected by gas chromatography. The reaction mixture (final volume 0.25 ml) contained (mM): Hepes-Na/KOH (pH 7.5), 20; $MgCl_2$, 5; ATP, 5; and an ATP-regenerating system consisting of 15 mM creatine phosphate and 25 μg creatine kinase (for details see [16]).

Partially purified nitrogenase (cf. [16]) was obtained by centrifugation of the heterocyst homogenate (1 mg chlorophyll/ml) at 100000 × *g* for 1 h, followed by centrifugation of the supernatant at 350000 × *g* for 5 h. The pellet, containing all nitrogenase activity, was resuspended in the same volume as the original heterocyst homogenate allowing nitrogenase activity, for better comparison, to be related to chlorophyll in assays not containing thylakoids (see section 3). The resuspension buffer consisted of (mM): Hepes-Na/KOH (pH 7.8), 30; Na/K-phosphate, 5; $MgCl_2$, 10.

3. RESULTS

As shown in fig.1A,B ferredoxin from heterocysts, reduced by either H_1 via hydrogenase (from *C. pasteurianum*) or by NADPH via $NADP^+$:ferredoxin oxidoreductase (*A. variabilis*), efficiently mediates electron flow to nitrogenase from *A. variabilis*, while ferredoxin from vegetative cells is completely inactive. Basal activities without added ferredoxin may be due to traces of heterocyst ferredoxin in the partially purified nitrogenase preparation obtained from heterocysts (see section 2).

Optical spectra of both ferredoxins exhibited the characteristic features of plant-type ferredoxins. Both were reduced by clostridial hydrogenase to the same extent (not shown). From this follows that the inefficiency of vegetative cell ferredoxin in reconstituting electron transport to nitrogenase is mainly due to poor interaction with the nitrogenase enzyme.

Inhibition of the basal rate in NADPH-dependent nitrogenase activity upon addition of low amounts of vegetative cell ferredoxin (fig.1B) may be explained by competition with trace amounts of heterocyst ferredoxin at the ferredoxin-binding site of $NADP^+$:ferredoxin oxidoreductase; complex formation between the latter enzyme and ferredoxin is well known [18–21]. Such a displacement does not become visible with ferredoxin reduced by clostridial hydrogenase (fig.1A).

When photoreduced by heterocyst thylakoids, with electrons from H_2 feeding into PS I via

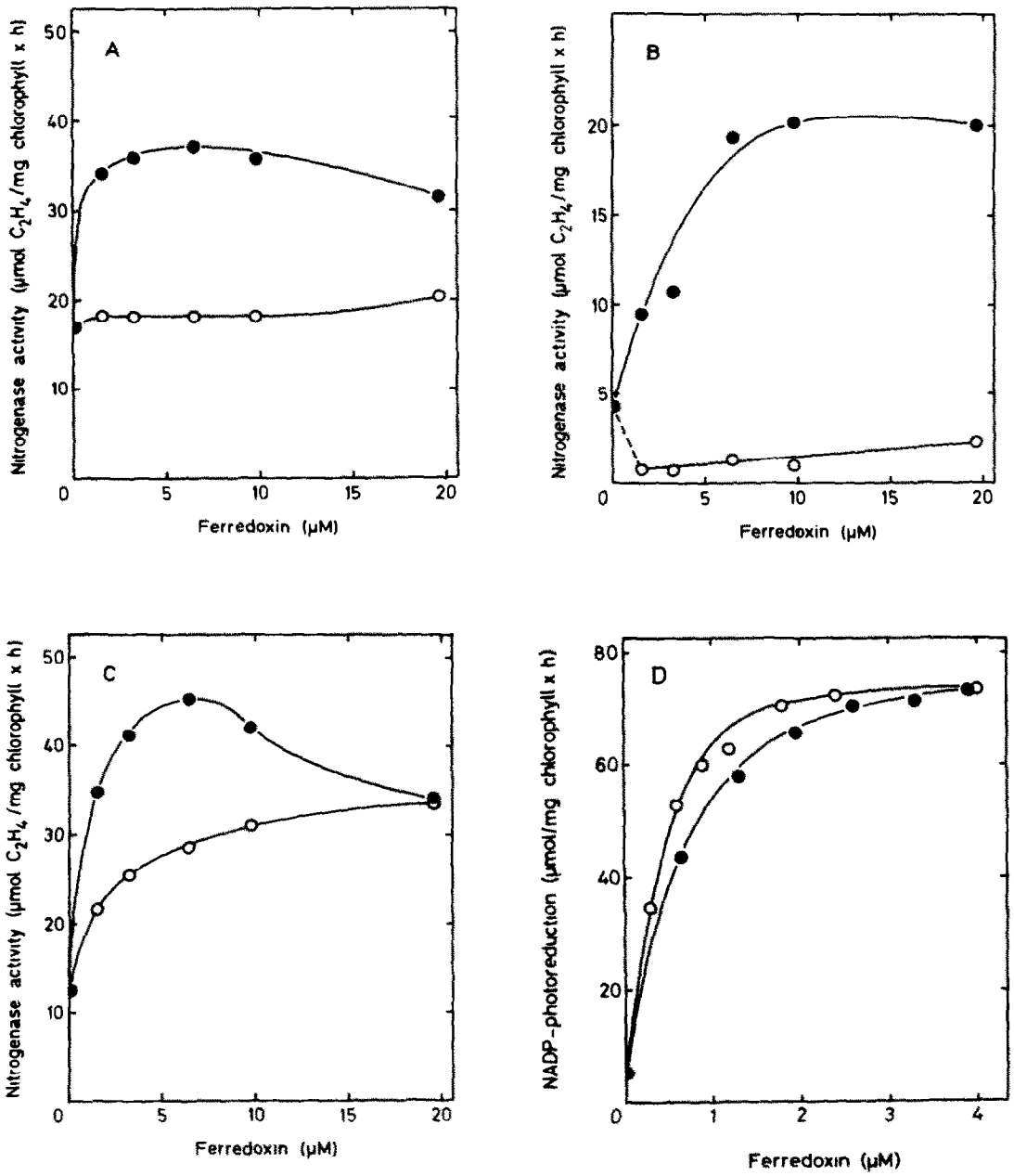

Fig.1. Reconstitution of different physiological electron transport systems by heterocyst ferredoxin (●) and by vegetative cell ferredoxin (○) from *A. variabilis*. Assays A–C each contained 25 μl of partially purified nitrogenase from *A. variabilis* heterocysts and an ATP-regenerating system in a final volume of 0.25 ml (see section 2), and additionally, (A) 5 μl clostridial hydrogenase; (B) 0.48 μM $NADP^+$: ferredoxin oxidoreductase and an NADPH-regenerating system consisting of 8 mM isocitrate, 60 μM $NADP^+$, 0.4 mg isocitrate dehydrogenase; (C) 25 μl washed heterocyst thylakoids (0.64 mg chlorophyll/ml) and 7.4 μM cytochrome *c*-553. For $NADP^+$ photoreduction (D) the reaction mixture contained in a final volume of 2.5 ml: 30 mM Hepes-Na/KOH, 5 mM Na/K-phosphate, 2 mM $MgCl_2$, 28 μl heterocyst thylakoids (0.88 mg chlorophyll/ml), 2 μM cytochrome *c*-553, 0.13 μM $NADP^+$: ferredoxin oxidoreductase and 0.5 mM $NADP^+$. The assays were conducted under H_2 (A,C,D) or argon (B). Light intensities were 700 μE/m^2 per s (C) and 980 μE/m^2 per s (D).

thylakoid-bound hydrogenase, both ferredoxins catalyze electron transfer to nitrogenase. Generation of a very low redox potential by PS I (< -550 mV) obviously allows ferredoxin from vegetative cells also to mediate electrons to nitrogenase, though only to a limited extent. In this system heterocyst ferredoxin again was much more active and maximal activity was achieved at comparably low concentrations. Higher concentrations ($\geq 10\ \mu M$) were inhibitory.

Both ferredoxins are equally active in reconstituting $NADP^+$ photoreduction by heterocyst thylakoids (fig.1D). Evidently there is no marked priority of either ferredoxin over the other in shuttling electrons between the PS I primary electron acceptor and $NADP^+$: ferredoxin oxidoreductase.

Altogether these data demonstrate the occurrence of a special ferredoxin synthesized in heterocysts, which is particularly designed to function as direct electron donor to nitrogenase. This view is further corroborated by the data of table 1. Nitrogenase activity in the dark, exhibited by (ferredoxin-containing) heterocyst homogenates upon addition of the electron donating substrate glucose 6-phosphate (cf. [15]) is further stimulated by about 50% after addition of heterocyst ferredoxin, whereas vegetative cell ferredoxin is completely inactive.

Table 1

Stimulation of nitrogenase activity in heterocyst homogenates by heterocyst ferredoxin as compared to ferredoxin from vegetative cells

Electron carrier	Concentration added (μM)		
	–	6.5	20
Heterocyst ferredoxin	29	45	40
Vegetative cell ferredoxin	29	29	31

The assays were conducted anaerobically (argon) in the dark. Besides an ATP-regenerating system it contained in a final volume of 0.25 ml: 2.5 mM glucose 6-phosphate as electron-donating substrate, 20 μM each NAD and $NADP^+$ as co-substrates, 25 μl of a heterocyst homogenate (1 mg chlorophyll/ml) and ferredoxin as indicated. Activities are given in μmol C_2H_4/mg chlorophyll per h

4. DISCUSSION

The presence of two species of ferredoxin in some cyanobacteria can be regarded as firmly established [5–13]. Several attempts have been made to assign a specific function in metabolism to one ferredoxin or the other [6,11–13]. Yet, no conclusive evidence has been obtained, whether different ferredoxins within one organism may operate in divergent biological reactions, such as $NADP^+$ photoreduction, cyclic photophosphorylation, light modulation of enzymes (in connection with thioredoxin), oxidative cleavage of pyruvate and in electron donation to nitrate and nitrite reductases, sulfite reductase, glutamate synthase, and to nitrogenase.

Isolation of ferredoxin from heterocysts, the site of nitrogen fixation in filamentous cyanobacteria, has not been reported yet. It is shown here that a distinct plant-type ferredoxin is present in these cells coupling effectively and specifically to nitrogenase, thus providing this enzyme with electrons potentially originating from different sources. These include soluble systems, such as NADPH via ferredoxin: $NADP^+$ oxidoreductase and probably pyruvate via pyruvate: ferredoxin oxidoreductase as well as PS I-activated electrons from H_2 and NADH [15,16,22,23]. Since heterocyst ferredoxin is as effective as vegetative cell ferredoxin in reconstituting $NADP^+$ photoreduction with heterocyst thylakoids (fig.1D), it is likely to replace the latter not only in electron donation to nitrogenase but also in other ferredoxin-requiring reactions of the heterocyst, e.g., the light-dependent transhydrogenase system [16,24].

In two former investigations on electron transport to nitrogenase (in a different context [16,24]) both ferredoxins were not used separately, since isolation of ferredoxin from whole filaments (comprising heterocysts) yielded a mixture of the two molecular species.

Besides effects due to protein-protein interaction, differences in redox potential, as reported for *Nostoc*, strain MAC ferredoxins I and II, could also contribute to the observed catalytic differences between heterocyst and vegetative cell ferredoxin. This may enable the (limited) activity of the latter as electron donor to nitrogenase when photoreduced by PS I at a very low redox potential.

Finally, the occurrence of two different ferredoxins in vegetative cells and heterocysts, which seem to be especially adapted to $NADP^+$ photoreduction and electron donation to nitrogenase, respectively, gives a plausible explanation for the clearly differential inhibition of these two basic physiological processes by the ferredoxin antagonist metronidazole reported for *A. cylindrica* [25].

A detailed comparative biochemical characterization of the two ferredoxins is in progress.

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