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Comparative characterization of ferredoxins from heterocysts and vegetative cells of *Anabaena variabilis*

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The isolation and purification of a ferredoxin from heterocysts of *Anabaena variabilis* (ATCC 29413) is described. Heterocyst ferredoxin differs from vegetative cell ferredoxin in amino-acid composition, molecular weight, midpoint redox potential, optical and EPR spectra and in its immunological properties. The data confirm the view of a new ferredoxin synthesized in heterocysts for specific interaction with nitrogenase.

Introduction

The presence of two species of ferredoxin in some cyanobacteria can be regarded as firmly established [1–11]. Differences in amino-acid composition [2–4,8,10,11] and redox potential [5] and less convincing differences in catalytic activity [2,4,7–9] have been reported. Recently we described the isolation and characterization of biological activities of ferredoxins isolated from heterocysts and vegetative cells of *Anabaena variabilis*. It was found that the ferredoxin from heterocysts efficiently and specifically coupled electron transport from different donor sources to heterocyst nitrogenase [12]. In the present report a detailed comparative biochemical characterization of ferredoxins from heterocysts and vegetative cells will be presented.

A preliminary report has been presented at the Fifth International Symposium on Photosynthetic Prokaryotes, Grindelwald, September 1985.

Materials and Methods

Photoautotrophic growth of *Anabaena variabilis* (ATC 29413) and isolation of heterocysts have been described [13,14]. Hydrogenase from *Clostridium pasteurianum* was prepared according to [15,16].

Heterocyst ferredoxins were obtained from French-press treated heterocysts as a light brown supernatant after centrifugation of the extracts at $350\,000 \times g$ for 5 h [12,14]. This supernatant was applied to a DEAE-Sepharose CL6B column (Pharmacia, 1.5×5 cm), equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was washed with 30 ml Tris buffer, containing 0.1 M NaCl, yielding heterocyst cytochrome *c*-553 in the eluate. Thereafter, the column was 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. Among other proteins, NADP : ferredoxin oxidoreductase appeared, then ferredoxin I (at about 0.32 M NaCl) clearly separated from ferredoxin II (at about 0.41 M NaCl). The major component (about 80%) was

Abbreviations: ATCC, American Type Culture Collection; Chl, chlorophyll; Fd, ferredoxin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

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ferredoxin I. Both ferredoxins were collected separately, diluted 1 : 4 with cold distilled water and rechromatographed on a DEAE-Sepharose CL6B column (1 × 3 cm), equilibrated with 20 mM Tris-buffer (pH 8.0). Concentrated ferredoxin I was eluted with 0.4 M NaCl and ferredoxin II with 0.5 M NaCl. As checked by SDS-polyacrylamide gel electrophoresis (17.5%, Ref. 17), both ferredoxins were essentially free of other proteins at this stage of purification. Contamination by polynucleotides could be removed by high-performance ion-exchange and gel chromatography. As a first step, a TSK 545 DEAE column was used (LKB Ultropac, 7.5 × 150 mm), equilibrated with Mops-NaOH (pH 7.0) at a flow rate of 0.5 ml/min. Ferredoxin was eluted by a linear gradient of 0–0.6 M NaCl; then 200 μl of the main fraction were directly applied to a TSK G200SW gel filtration column (LKB Ultropac, 7.5 × 600 mm), equilibrated with 20 mM Mops-NaOH (pH 7.0)/0.4 M NaCl, at a flow rate of 0.2 ml/min. Ferredoxin eluted as a sharp peak after the contaminating nucleotides. For isolation of vegetative cell ferredoxin essentially the same procedures were used. Diluted samples of ferredoxin II (and vegetative cell ferredoxin) showed a tendency to denaturation, apparent as a shift of the 330 nm absorbance peak to 325 nm, with a shoulder at 308 nm. Prolonged storage at −30°C eventually led to decolorization of the ferredoxin.

For determination of molecular weight, SDS-polyacrylamide gels (17.5%) containing 4 M urea were used. The ferredoxin samples (about 5 μg protein) were incubated at 37°C for 1 h with 50 mM iodoacetamide and protease inhibitors were added (1 mM phenylmethylsulfonyl fluoride and 1 mM *p*-aminobenzamidine). Thereafter, the carboxymethylated ferredoxins were heated for 5 min at 95°C in the presence of 2% SDS and 1% dithiothreitol. For molecular weight calibration the following standards were used (from Sigma): bovine serum albumin (66 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), soybean trypsin inhibitor (20 100) and lactalbumin (14 200). Additionally, molecular weight was determined in a nondenaturing gel system by the use of 'Ferguson plots' [18]. In this procedure proteins are electrophoresed on gels of various polyacrylamide concentrations (17.5–25%). The logarithms of the retardation coefficients were plotted against the logarithms of molecular weight. The following proteins yielded a fairly straight line: bovine serum albumin, ovalbumin, soybean trypsin inhibitor and cytochrome *c*-553 from *Scenedesmus acutus* (10 300). Molecular weight by gel-filtration was determined with the TSK G2000 SW column, equilibrated with 0.1 M potassium phosphate buffer, (pH 6.8)/0.1 M NaCl. Calibration standards were (from LKB): bovine serum albumin, ovalbumin, myoglobin (17 000), ribonuclease A (13 700) and aprotinin (6500).

Isoelectric points were determined in ultrathin polyacrylamide gels (Servalyt-precotes, pH 3–6) by horizontal flat-bed electrophoresis (LKB, Multiphor), using the following proteins (from Pharmacia) for calibration (p*I*): pepsinogen (2.8), amyloglucosidase (3.5), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.2). Due to the low buffering capacity of the ampholines used, p*I* values below 2.7 could not be determined accurately.

Amino-acid analysis was performed according to standard methods [20] on a Biotronik Amino-Acid Analyzer LC 5000. The proteins were hydrolyzed for 24, 48 and 72 h with 6 M HCl, containing 0.1% phenol, at 110°C, using sealed, evacuated Pyrex tubes. Protein concentration of ferredoxins was calculated using the molar extinction coefficient at 422 nm of 9700 $M^{-1} \cdot cm^{-1}$.

Optical absorption spectra were obtained by a Shimadzu UV 350 spectrophotometer. EPR-spectra were recorded on a Bruker B-ER 420 instrument, equipped with a Helitran LTD-3-110 system at 12 K, with a microwave power of 1.6 mW and a frequency of 9.31 GHz. The ferredoxin samples (about 0.1 mM in 50 mM Tris-HCl (pH 8.0)) were reduced with dithionite at room temperature, before being frozen in liquid nitrogen.

Midpoint redox potentials were determined by plotting the absorbance change at 422 nm upon reduction of the ferredoxins by the system H_2/hydrogenase (*C. pasteurianum*) at different pH values. The following buffers were used (0.1 M): Tris-HCl (pH 8.5 and 8.0); Hepes-NaOH (pH 7.5); Mops-NaOH (pH 7.0); and Tes-NaOH (pH 6.5). Redox potentials were calculated using the

Nernst equation. 5 µl of hydrogenase (3500 U/ml) were added anaerobically to a cuvette, closed by a septum stopper, containing ferredoxin (about 12 µM) in hydrogen-saturated buffers; spectra were recorded until equilibrium was reached.

Immunological tests. Double-diffusion tests were carried out in agarose gels (1.0%, dissolved in 0.05 M veronal buffer (pH 8.6), see Ref. 21). Electrophoretic transfer of proteins, separated on SDS-urea polyacrylamide gels, onto nitrocellulose sheets (0.45 µm pore size) and subsequent detection of protein-antibody complexes by peroxidase conjugated goat anti-rabbit immunoglobulin G (immunoblotting) was performed according to Refs. 22 and 23. Due to the low molecular weight and high charge of ferredoxins, transfer (45 min at 48 V, 500 mA) led to the appearance of some ferredoxin on the back of the nitrocellulose sheet. Therefore this method could not be used for quantitative comparison.

The procedures for preparation of antisera from rabbits against spinach ferredoxin and ferredoxin from vegetative cells of *Anabaena* are described in Ref. 24. For immunoblotting experiments the sera were diluted 1 : 600 in serum buffer (cf. Ref. 23).

Results and Discussion

The absorption spectra of the two heterocyst ferredoxins (I and II) are shown in Figs. 1A and B. The spectrum of ferredoxin II is identical to that of vegetative cell ferredoxin (Fd v), with the usual absorbance maxima at 463, 421/422, 330 and 276 (284) nm. Heterocyst-ferredoxin I can be distinguished by a red shift to 466 (and 331) nm and an ultraviolet absorbance maximum at 274 nm. The fine structure seen at 282, 268, 258 nm is probably due to the lower content of tyrosine residues (see below). Similarly the maximum absorbance ratio (A_{422}/A_{275}) was higher for ferredoxin I (0.74), than for the other two ferredoxins (0.6). The reduced forms (by H_2/hydrogenase) showed weak absorbance maxima at 540, 469, 396, (340), 307 nm (Fd I) and at 538, 471, 399, (345), 312 nm for Fd v (see Fig. 2A). The EPR spectra were again identical for vegetative cell ferredoxin and ferredoxin II with principal g values at 1.88 (g_x), 1.96 (g_y) and 2.05 (g_z). A shift in the g_x region was observed with heterocyst ferredoxin I (g_x, 1.9; g_y, 1.95; g_z, 2.0455), possibly reflecting a different geometry around the iron-sulfur center (cf. Ref. 8). The rhombic EPR-signals of the reduced proteins are characteristic of a plant-type ferredoxin with a two-iron/two-sulfur cluster.

Determination of molecular weights by different methods yielded somewhat different values. Molecular sieving revealed single symmetrical peaks with estimated molecular masses of 21.5 kDa for ferredoxin I and 23.3 kDa for the other two ferredoxins. Polyacrylamide gel electrophoresis in the presence of SDS/urea yielded single bands with apparent molecular masses of 23.5 kDa (Fd I) and 27 kDa (Fd II, Fd v). Obviously, dimerization of the ferredoxins could not be prevented under these conditions (cf. Ref. 6). However, in nondenaturating gradient gels (10–22.5%), the ferredoxins moved close to the bromophenol marker front, indicating highly charged proteins of low molecular weight. Ferguson plots [18,19] again showed slight differences in molecular mass of 11.5 kDa for ferredoxin I and 13 kDa for the other two proteins.

The higher apparent molecular weights of ferredoxin II and ferredoxin v indicated that some ionic interaction with the gel matrix could not completely be prevented, due to the very low isoelectric points. This was confirmed by isoelectric focusing. Values of pH 3.0 for heterocyst ferredoxin I and below pH 2.7 (about 2.4) for the other two ferredoxins were obtained. The midpoint redox potentials (E_m) were determined by reductive titration with the system H_2/hydrogenase. An example of ferredoxin I reduction is shown in Fig. 2A. The E_m value for heterocyst ferredoxin I was -405 mV (± 10 mV) and for vegetative cell ferredoxin -433 mV (± 10 mV), assuming a pH-independent, one-electron process (Fig. 2B).

The results of amino-acid analysis of all three ferredoxins from *A. variabilis* are summarized in Table I. The amino-acid composition of heterocyst ferredoxin I compared to the other two showed clear differences, with main changes occurring in the amounts of following amino-acid residues: the Asx, Ala and Tyr content was decreased, whereas the Glx, Ile and Lys content was increased per mole of ferredoxin I. The higher isoelectric point of heterocyst ferredoxin I is explained by additional Lys(2) and Arg(1) residues and additional

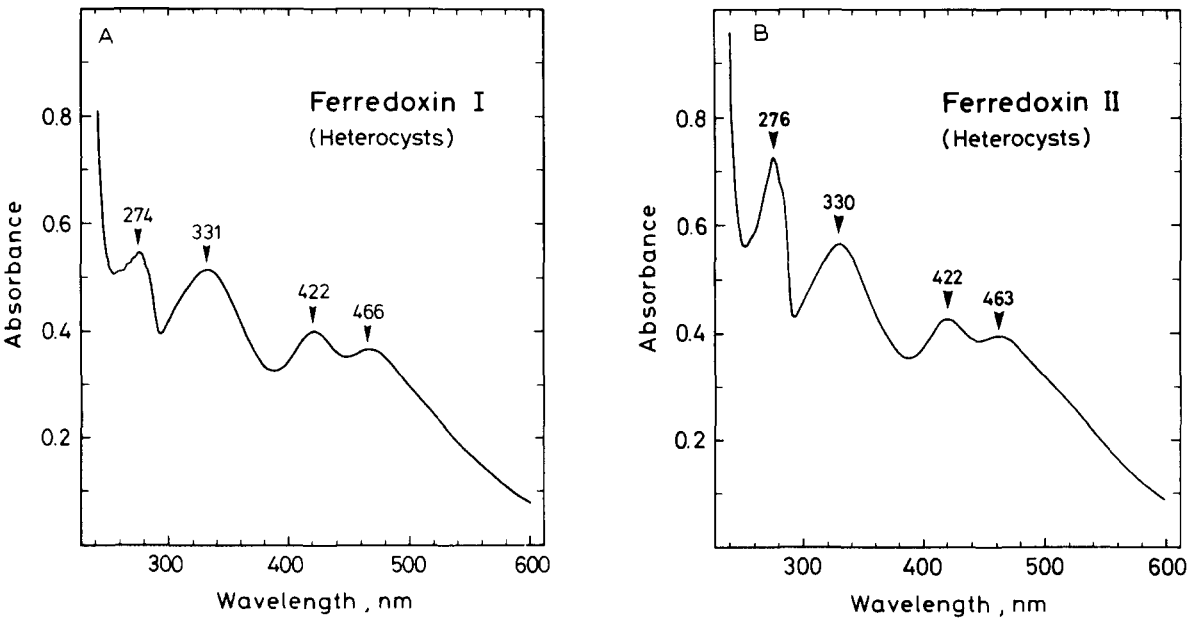

Fig. 1. Absorption spectra of ferredoxins I (A) and II (B) from heterocysts of *A. variabilis*.

NH_3 released. Comparison of heterocyst ferredoxin I and vegetative cell ferredoxin by the method of Marchalonis and Weltman [25] yielded an *SΔQ*-value of 63.6. This is in the range of *SΔQ* values calculated for ferredoxins I and II from *Nostoc verrucosum* [11], *Aphanothece sacrum* [10],

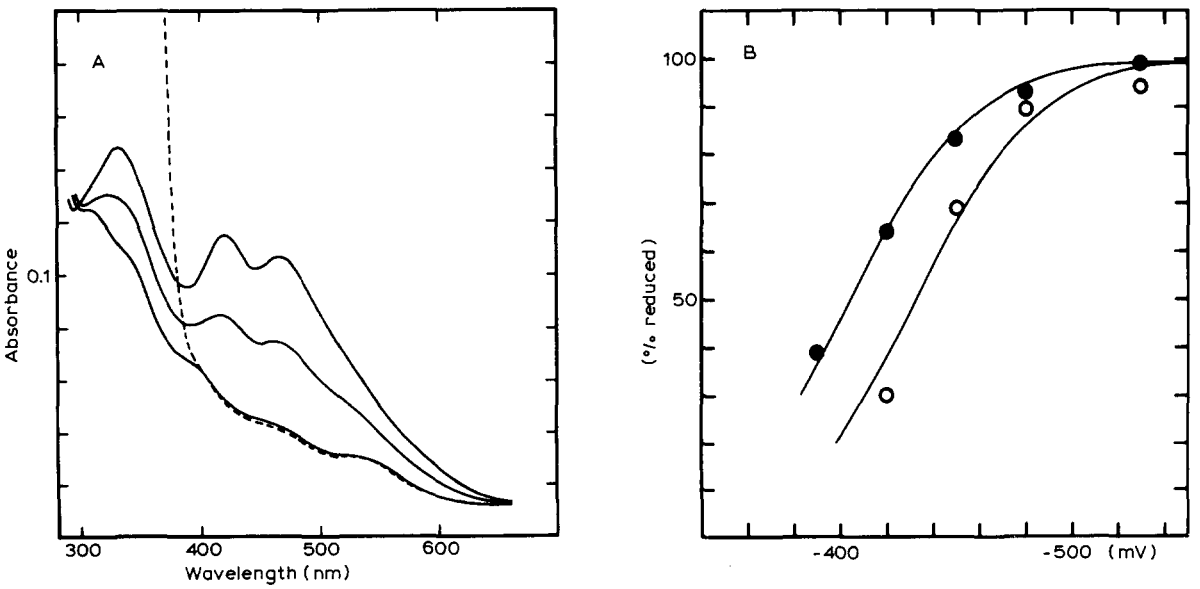

Fig. 2. (A) Reduction of heterocyst ferredoxin I by H_2/hydrogenase (*C. pasteurianum*) at different pH values. Upper trace: (oxygen)-oxidised ferredoxin; middle and lower trace: reduction by H_2/hydrogenase at pH 6.5 and 8.5, respectively; dotted line: reduction by 0.25 mM dithionite. (B) Reductive titration of heterocyst ferredoxin I (●) and vegetative cell ferredoxin (○) by H_2/hydrogenase (*C. pasteurianum*) at different pH values. The solid lines are theoretical plots of the Nernst equation for a one-electron carier with assumed midpoint potentials of −405 mV (●) and −433 mV (○), respectively.

TABLE I

AMINO-ACID COMPOSITION OF FERREDOXINS FROM HETEROCYSTS AND VEGETATIVE CELLS OF *A. VARIABILIS*

n.d., not determined.

Amino-acid residue	Heterocyst		Vegetative cell ferredoxin
	ferredoxin I	ferredoxin II	
Asx	11	14	14
Thr	8	9	9
Ser	7	6	6
Glx	18	14	14
Pro	3	3	3
Gly	8	6	7
Ala	4	8	8
Cys [a]	4	4	4
Val	7	7	7
Met	0	0	0
Ile	8	5	5
Leu	7	7	7
Tyr	3	5	5
Phe	3	3	3
His	2	1	1
Lys	5	3	3
NH_3	12	11	11
Arg	2	1	1
Trp	n.d.	n.d.	n.d.
Number of residues	100	96	97
Calculated molecular weight (apoprotein)	10998	10452	10509

[a] Minimum amount as assumed from 2Fe/2S-cluster of plant-type ferredoxins.

and *Nostoc*, strain MAC [11], with values of 43.8, 48.3 and 82.2 respectively, showing only a fairly close relationship between these proteins (*SΔQ* values above 100 indicate unrelated proteins). The calculated molecular weight of 11000 for heterocyst ferredoxin I corresponded closely to the determinations described above, whereas the calculated molecular weights of ferredoxin II and vegetative cell ferredoxin were considerably lower (about 10500) than those obtained by gel-filtration or polyacrylamide gel electrophoresis. The properties of all three ferredoxins are summarized in Table II.

Immunological studies further supported the notion that ferredoxin I from heterocysts is quite different from the other two ferredoxins. Using a

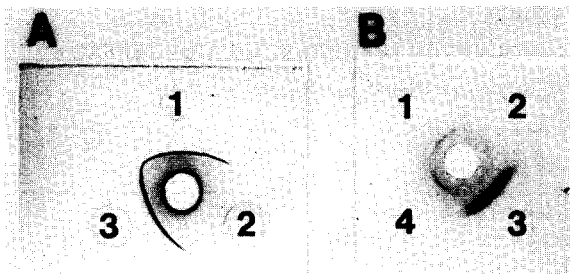

Fig. 3. Double-diffusion test of antisera against (A) vegetative ferredoxin and (B) spinach ferredoxin. The center well contained the respective antisera (20 μl) (A) the three outer wells contained 1.5 μg of vegetative cell ferredoxin (1), heterocyst ferredoxin I (2) and II (3). (B) The four outer wells contained each 0.2 μg of vegetative cell ferredoxin (1), heterocyst ferredoxin I (2), spinach ferredoxin (3) and heterocyst ferredoxin I (4). Diffusion period: 16 h at 8°C.

double-diffusion test, no cross-reaction of heterocyst ferredoxin I with an antiserum against vegetative cell ferredoxin was observed. Interestingly, an

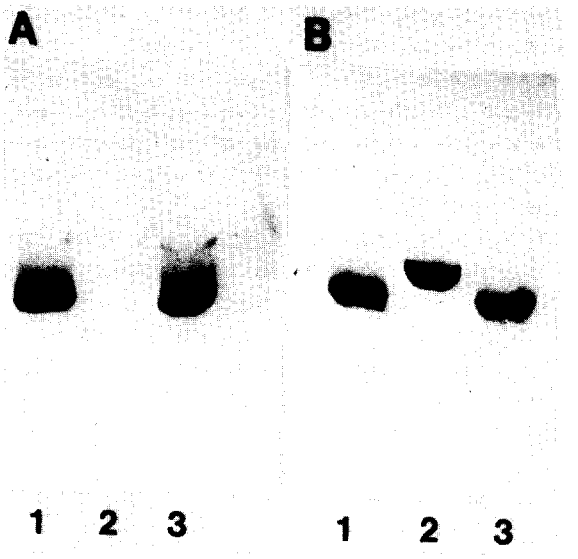

Fig. 4. Detection of ferredoxins from heterocysts and vegetative cells of *A. variabilis* by immunoblotting. After electrophoretic transfer from SDS/urea polyacrylamide gels onto nitrocellulose sheets, the proteins were detected by incubation with antiserum against (A) vegetative cell ferredoxin and (B) spinach ferredoxin. Protein-antibody complexes were identified by peroxidase-conjugated goat anti-rabbit IgG. The lanes contained from left to right: (A) 0.2 μg of vegetative cell ferredoxin (1), heterocyst ferredoxin I (2) and II (3). (B) 0.8 μg of vegetative cell ferredoxin (1), heterocyst ferredoxin I (2) and II (3).

TABLE II

PROPERTIES OF FERREDOXINS ISOLATED FROM HETEROCYSTS AND VEGETATIVE CELLS OF *A. VARIABILIS*

PAGE, polyacrylamide gel electrophoresis.

	Heterocyst ferredoxin I	Heterocyst ferredoxin II/ vegetative cell ferredoxin [a]
Molecular mass (kDa):		
(A) SDS/4 M urea PAGE	23.5	27
(B) Nondenatured PAGE	11.5	13
(C) Gel filtration	21.5	23.3
(D) Amino-acid analysis	11.0	10.5
Optical absorbance maxima (nm)		
(a) oxidized	466, 422, 331, (282), 274	463, 422, 330 (282), 276
(b) reduced	540, 469, 396, (340), 307	538, 471, 399, (345), 312
Radio A_{422}/A_{275}	0.74	0.6
Isoelectric point (pH)	3.0	< 2.7
EPR spectra (12 K) g_x, g_y, g_z	1.90, 1.95, 2.045	1.88, 1.96, 2.05
Midpoint redox potential (±10 mV)	−405 mV	−433 mV

[a] The data for vegetative cell ferredoxin are identical to those for heterocyst ferredoxin II.

anti-spinach ferredoxin antiserum reacted weakly with heterocyst ferredoxin as well (Fig. 3). These results were confirmed by immunoblotting experiments. Although the gel was overloaded with protein on purpose, no cross-reaction of heterocyst ferredoxin I with antiserum against vegetative cell ferredoxin was observed. Again, anti-spinach ferredoxin antiserum reacted with all three *Anabaena* ferredoxins (Fig. 4).

The results presented above clearly show that in heterocysts a new ferredoxin is synthesized, reacting specifically with heterocyst nitrogenase [12]. Compared to the ferredoxin of vegetative cells, it can be clearly distinguished by its biochemical and biophysical properties, which are reflected by a different amino-acid composition. The approx. 27 mV higher redox potential of heterocyst ferredoxin I would make this protein more easily reducible by carbohydrate metabolism via the NADPH/NADP: ferredoxin oxidoreductase system. By specific interaction with nitrogenase, electrons would be preferably channeled into nitrogen fixation. We further speculate that the minor component of the two heterocyst ferredoxins may function in cyclic photophosphorylation, remaining associated with Photosystem I (cf. Ref. 24) and contributing to the energy supply of the nitrogenase reaction.

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