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TWO PLANT-TYPE FERREDOXINS FROM A BLUE-GREEN ALGA, *NOSTOC VERRUCOSUM*

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SUMMARY

Two plant-type ferredoxins were isolated and purified from a blue-green alga, *Nostoc verrucosum*. They were separable by chromatography on a DEAE-cellulose column. The slow-moving band was designated ferredoxin I (Fd I) and the fast-moving band was ferredoxin II (Fd II). The ratio of the yield of ferredoxins I and II was about 1 : 0.84. Both ferredoxins had absorption spectra similar to those of plant-type ferredoxins. Two atoms of non-heme iron and two of labile sulfur were found per mol of both ferredoxin I and ferredoxin II. Their molecular weights were identical and estimated to be about 18 000 by a gel filtration method. The biochemical activities of these *Nostoc* ferredoxins were studied: the NADP photoreduction activity on one hand and the NADP-cytochrome *c* reductase activity on the other.

INTRODUCTION

Since Black et al. [1] first demonstrated the presence of ferredoxin in blue-green algae in 1963, the plant- or chloroplast-type ferredoxins have been isolated and purified from several genera of the division, *Anabaena* [2], *Anacystis* [3], *Nostoc* [4], *Spirulina* [5], *Microcystis* [6] and *Aphanothece* [7].

Among the studies on ferredoxin, one of recent interest shows that two different molecular species of ferredoxin have been detected in one organism. Two such species of ferredoxin have been found in a facultative photosynthetic bacterium, *Rhodospirillum rubrum* [8], a nitrogen-fixing obligate aerobic bacterium, *Azotobacter vinelandii* [9], a nitrogen-fixing bacterium, *Bacillus polymyxa* [10], and higher plants, *Leucaena glauca* [11] and *Phytolacca americana* [12]. In blue-green algae, two types of ferredoxin have been reported, in *Aphanothece sucrum* (as major and minor components [7, 13]), and in *Nostoc* Strain MAC (as types I and II [14]).

This article presents more evidence for the coexistence of two different ferredoxins in a blue-green alga, *Nostoc verrucosum*. The biochemical properties of these two ferredoxins, ferredoxins I and II, are also described.

MATERIALS AND METHODS

Nostoc cells

Colonies of *Nostoc verrucosum* ('Ashitsuki' in Japanese) were collected from the surface of the submerged rocks at the Sumiyoshi River in Mt. Rokko, Kobe. They were washed with water to remove contaminants and stored as frozen cells. The washed colonies were composed of uniform *Nostoc* filaments and no contaminants could be detected in them microscopically.

Purification of ferredoxins from Nostoc cells

Extraction and isolation. 2 kg of frozen cells were thawed and then 100 g NaCl and 1 g Tris-hydroxymethylaminomethane were added. The mixture was left in the cold room (10 °C) overnight. During this treatment, ferredoxins could be extracted without mechanical aid, together with a violet substance. The violet extracts were obtained by squeezing the algal mixture through a cloth by a hand press. The algal debris were reextracted with 1 l of 5 % NaCl solution. The combined extracts were fractionated by ammonium sulfate at 70 % saturation in order to precipitate the violet substances. The brownish pink supernatant solution was directly passed through a DEAE-cellulose column (3×10 cm) equilibrated with 70 % saturation solution of ammonium sulfate neutralized by ammonia, according to the description of Mayhew [15]. In spite of the high concentration of ammonium sulfate, ferredoxin could be adsorbed on the cellulose column. The column was then washed with the same ammonium sulfate solution and the ferredoxins were eluted with 0.1 M Tris · HCl buffer, pH 7.5, containing 0.8 M NaCl.

Separation of ferredoxins I and II. The crude ferredoxins were diluted three times with water and loaded onto the first DEAE-cellulose column (2×30 cm) equilibrated with 0.02 M Tris · HCl buffer, pH 7.5, containing 0.35 M NaCl. The brownish red band initially adsorbed to the top of the column was split into two red bands during the chromatography. The development of the column was continued with the same buffer solution, until the fast-moving red band, ferredoxin II, was completely eluted out from the column. The NaCl concentration in the buffer was then increased up to 0.38 M. Subsequently, the slow-moving band, ferredoxin I, was moved down faster and eluted from the column.

Further purification of ferredoxins I and II. The ferredoxin II fraction from the previous column was diluted 2.5 times with water and collected on a small DEAE-cellulose column (2×3 cm) equilibrated with 0.01 M Tris · HCl buffer, pH 7.5, containing 0.08 M NaCl. The red DEAE-cellulose was suspended in the same buffer and again placed on the top of the second DEAE-cellulose column (2×30 cm) equilibrated with 0.02 M Tris · HCl buffer, pH 7.5, containing 0.35 M NaCl. The main fraction of ferredoxin II was concentrated by a small DEAE-cellulose column and then applied to the Sephadex G-75 column equilibrated with the same Tris · HCl buffer containing 0.33 M NaCl.

Further purification of ferredoxin I was performed in the same manner as in the case of ferredoxin II, except that 0.38 M NaCl was used on the DEAE-cellulose column chromatography instead of 0.35 M NaCl for ferredoxin II.

Estimation of the Nostoc ferredoxins

The ferredoxins were estimated by measuring absorbance at 420 nm for crude preparations in an ordinary way. Purified ferredoxins I and II were estimated at 425 and 422 nm by using millimolar coefficients of 11.51 $mM^{-1} \cdot cm^{-1}$ and 11.13 $mM^{-1} \cdot cm^{-1}$, respectively.

Determination of non-heme iron and labile sulfur contents

The non-heme iron content was determined with a Shimadzu atomic absorption/flame spectrophotometer, Model AA-610S. The standard solution of ferric chloride, 1000 ppm solution, was purchased from Nakarai Chemical Co. The ferredoxin solution was directly sprayed to the flame without ashing treatments.

The labile sulfur content was determined by the method improved by Suhara et al. [16]. Spinach ferredoxin was used as the control.

Determination of molecular weights

The molecular weights of ferredoxins I and II were estimated by gel filtration on a Sephadex G-75 column (2×50 cm) according to the method of Andrews [17]. Ovalbumin, chymotrypsinogen A, myoglobin and cytochrome *c* were used as markers. The moving phase consisted of a solution, 0.05 M Tris · HCl buffer, pH 7.5, containing 0.1 M NaCl.

Biochemical activities of Nostoc ferredoxins

The photosynthetic activity of *Nostoc* ferredoxins was determined by NADP photoreduction by using spinach broken chloroplasts. According to the method described by Asada and Takahashi [18], whole spinach chloroplasts were prepared with an isotonic solution containing 0.4 M sucrose, 50 mM tricine-KOH buffer, pH 8.0, and 10 mM NaCl. Broken chloroplasts were prepared from the whole chloroplasts essentially by the procedure described by Whatley and Arnon [19], except for the use of a hypotonic solution (ten-fold dilution of the above isotonic solution). The reaction mixture (2 ml) contained broken chloroplasts (about 40 μg chlorophyll), 25 mM tricine-KOH buffer, pH 8.0, 0.1 mM NADP and *Nostoc* ferredoxins. Reduction of NADP was determined by the increase in absorbance at 340 nm.

NADPH-cytochrome *c* reductase activity was determined by the reaction system which was constituted of 50 mM Tris · HCl buffer, pH 7.5, 0.1 mM NADPH, 0.04 mM cytochrome *c*, 0.226 units of *Nostoc* or spinach ferredoxin-NADP reductase (reduced NADP : ferredoxin oxidoreductase, EC 1.6.7.1) and *Nostoc* ferredoxins in 1 ml. Spinach ferredoxin-NADP reductase was prepared by the method described by Shin [20]. The *Nostoc* enzyme was prepared from the precipitates of the extracts by 70 % saturation of ammonium sulfate (see Extraction and isolation). Details of the purification procedures will be published elsewhere. One enzyme unit of ferredoxin-NADP reductase was defined as the amount of enzyme which causes a decrease in absorbance of 1.0 per min at 600 nm, due to reduction of 2,6-dichlorophenol indophenol by the NADPH diaphorase activity of the flavoprotein [21].

Biochemicals

NADP and NADPH were purchased from Oriental Yeast Co., Ltd. Horse heart cytochrome *c* was from Sigma Chemical Co. The ovalbumin, chymotrypsinogen

A, myoglobin and cytochrome *c* used as markers for gel filtration were obtained from Schwarz BioResearch, Inc.

RESULTS AND DISCUSSION

Purification of Nostoc ferredoxins

Nostoc ferredoxins were easily extracted from the frozen cells with a buffered saline solution as in the case of *Aphanothece* ferredoxin [7]. For the extraction, the freezing and thawing process was indispensable and the addition of 5 % NaCl was very effective.

Ammonium sulfate fractionation was extremely effective in the purification of ferredoxin. It removed many impurities from the extracts, including phycobilins. The ferredoxins remained in the supernatant of 70 % saturated ammonium sulfate. Usually, the added ammonium sulfate had to be removed from the supernatant by dialysis prior to the DEAE-cellulose treatment. In the preparation method, however, ferredoxins in the supernatant were directly adsorbed on a DEAE-cellulose column equilibrated with 70 % saturated ammonium sulfate. This improved procedure was advantageous not only for saving time during dialysis but also for minimizing denaturation of ferredoxins, because ferredoxins were unstable in the solution at low salt concentrations.

The crude ferredoxins contained two kinds of ferredoxin, ferredoxins I and II which were clearly separated into two red bands on the first DEAE-cellulose column as shown in Fig. 1. The ratio of the amounts of ferredoxins I and II was almost constant in the *Nostoc* cells at different seasons. In Table I, the yields of the two ferredoxins obtained from the first DEAE-cellulose column were indicated. 1 kg of cells contained about 0.64 μmol of ferredoxin I and 0.54 μmol of ferredoxin II. The ratio of the two ferredoxins was 1 : 0.84.

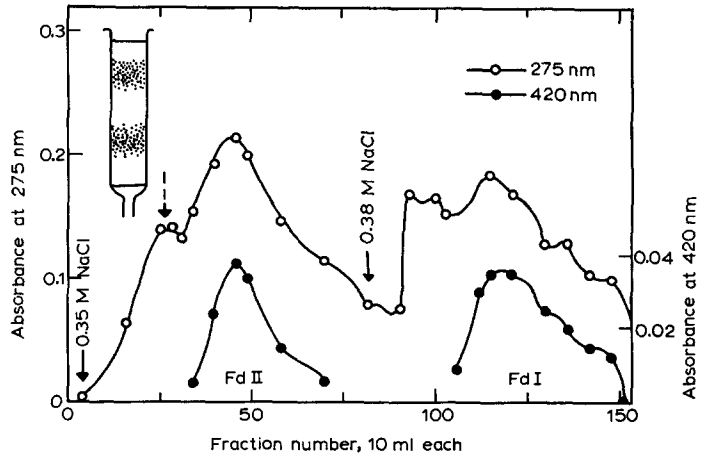

Fig. 1. Separation of ferredoxins I and II by the first DEAE-cellulose column. The crude ferredoxin adsorbed on the top of DEAE-cellulose column (2 × 30 cm) was chromatographed with 0.02 M Tris · HCl buffer, pH 7.5, containing 0.35 M NaCl. NaCl concentration was then changed to 0.38 M. ←, indication of NaCl concentration. Inset sketch shows the separation of ferredoxins I and II on the column at the stage indicated by broken arrow, ← – –. Fd, ferredoxin.

TABLE I

YIELD OF THE TWO FERREDOXINS FROM THE FIRST DEAE-CELLULOSE COLUMN

Fd I, ferredoxin I; Fd II, ferredoxin II.

Date of collection	Amount of cells used (kg)	Fd I (μmol)	Fd II (μmol)	Fd I : Fd II
May, 1974	2	1.13	0.95	1 : 0.84
Nov., 1974	1.86	0.91	0.79	1 : 0.87
May, 1975	2	1.71	1.42	1 : 0.83
Mean	1	0.64	0.54	1 : 0.84

Further purification of ferredoxins I and II were performed separately by successive chromatographies on the second DEAE-cellulose column and Sephadex G-75 column. During the purification of the ferredoxins, the absorbance ratios, $A_{420\ nm}/A_{275\ nm}$ and $A_{260\ nm}/A_{275\ nm}$ were measured as the index of purity. Although DEAE-cellulose column chromatography was an effective procedure for removal of impure proteins, it did not improve the absorbance ratio, $A_{420\ nm}/A_{275\ nm}$ so extensively. The phenomenon appeared to be due to absorbance of contaminating nucleotide-like substances which moved together with ferredoxins on the DEAE-cellulose column. It was found that the contaminating nucleotides were effectively removed by chromatography on a Sephadex G-75 column with Tris · HCl buffer containing a high concentration of NaCl. The elution pattern of ferredoxin II from the Sephadex G-75 column is shown in Fig. 2. The contaminating nucleotides eluted from the column immediately after the ferredoxin II fraction. This was also the case in the purification of ferredoxin I. When the nucleotides were not completely removed, Sephadex chromatography was repeated. After the second Sephadex G-75 column chromato-

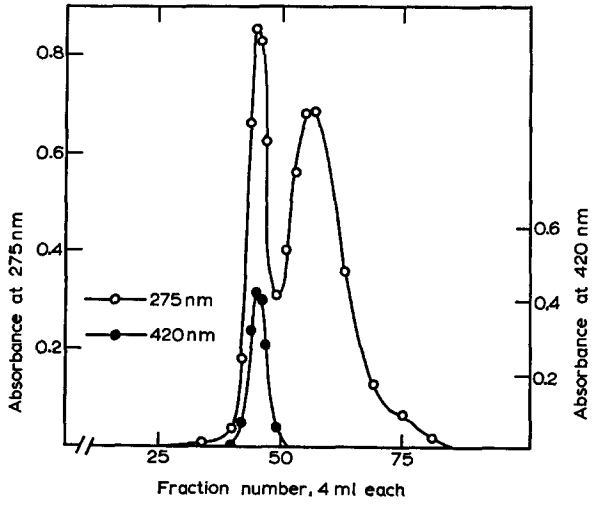

Fig. 2. Removal of nucleotides from ferredoxin II preparation by Sephadex G-75 column. Sephadex G-75 column (2 × 100 cm) was equilibrated with 0.02 M Tris · HCl buffer, pH 7.5, containing 0.33 M NaCl.

TABLE II

PURIFICATION PROCESS OF *NOSTOC* FERREDOXINS

Purification steps	Volume (ml)	Amount (E_{420} × ml)	$\frac{420}{275}$	$\frac{260}{275}$
Nostoc cells	2 (kg)			
Extract	2750			
Crude ferredoxin	38.5	(78.93)	0.06	1.20
Ferredoxin I				
1st DEAE-cellulose	14.5	13.05	0.15	1.25
2nd DEAE-cellulose	6.2	8.56	0.26	1.17
1st Sephadex G-75	28.0	6.16	0.44	0.99
2nd Sephadex G-75	20.0	4.40	0.50	0.99
Peak fraction*	4.0	1.16	0.52	0.97
Ferredoxin II				
1st DEAE-cellulose	22.0	10.56	0.11	1.32
2nd DEAE-cellulose	6.2	8.74	0.15	1.23
1st Sephadex G-75	24.0	6.76	0.46	0.96
2nd Sephadex G-75	30.0	3.90	0.49	0.91
Peak fraction*	10.0	1.50	0.50	0.90

* The measurements were carried out at 425 nm for ferredoxin I and 422 nm for ferredoxin II instead of at 420 nm.

graphy, the absorbance ratio of ferredoxin I was 0.52 and that of ferredoxin II was 0.50, as summarized in Table II.

Physico-chemical properties of Nostoc ferredoxins I and II

Nostoc ferredoxins showed absorption spectra very similar to those of plant- or chloroplast-type ferredoxins, as indicated in Fig. 3. Ferredoxin I had absorption peaks at 465, 425, 332 and 276 nm with a shoulder at 282 nm. The spectral shape was similar to those of *Nostoc* ferredoxin so far reported [4] and of the major component of *Aphanothece* ferredoxin [7, 13]. On the other hand, ferredoxin II showed absorption peaks at 466, 422, 331 and 278 nm, with shoulders at 281 and 290 nm. It resembled the minor component of *Aphanothece* ferredoxin [13] and a typical plant-type spinach ferredoxin. The distinct differences on absorption spectra in ultra-violet regions between the two ferredoxins suggest that the two ferredoxins have different amino acid compositions. Differences in amino acid composition have been demonstrated between the two *Aphanothece* ferredoxins [13].

Table III shows the results of the atomic absorption spectrophotometrical analysis of non-heme iron in ferredoxins I and II. The non-heme iron analyzed can by expressed as shown by Eqn. 1.

$$\text{Fe(ppm)} = n\ 55.84 \times \frac{A}{E} \qquad (1)$$

A : absorbance of ferredoxin used
E : molar extinction coefficient
n : gatoms of iron molecule

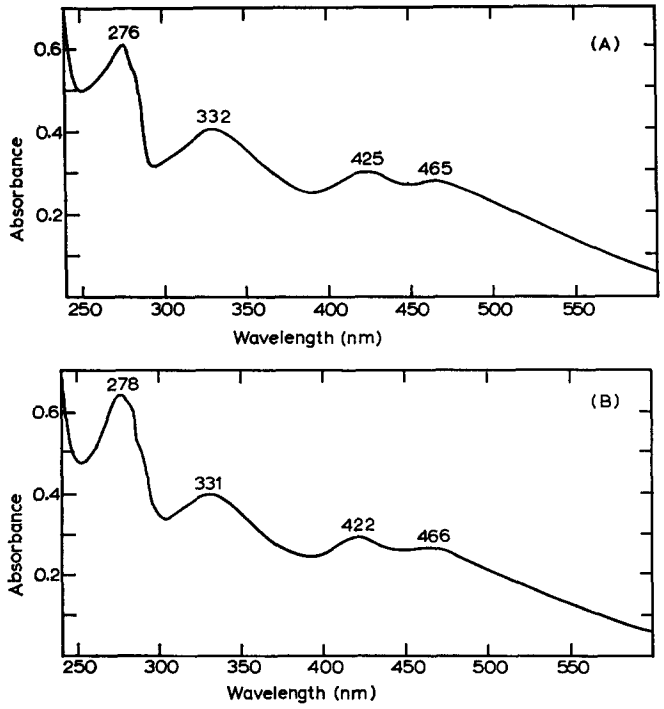

Fig. 3. Absorption spectra of *Nostoc* ferredoxins. The spectra of the ferredoxins were measured in 0.02 M Tris · HCl buffer, pH 7.5, containing 0.33 M NaCl with a Shimadzu multipurpose recording spectrophotometer, Model MPS-5000. (A), ferredoxin I; (B), ferredoxin II.

When there were assumed to be two atoms of iron per mol of ferredoxin, the molar extinction coefficients were calculated to be $11.51 \cdot 10^3$ for ferredoxin I and $11.13 \cdot 10^3$ for ferredoxin II. These values were reasonable for plant-type ferredoxins.

Upon analysis, labile sulfur in ferredoxins I and II was found to be 2.16 and 2.30 per mol, respectively. It is, therefore, possible to conclude that one molecule of each *Nostoc* ferredoxin contains two atoms of iron and two of labile sulfur.

TABLE III

DETERMINATION OF IRON CONTENT AND MOLAR EXTINCTION COEFFICIENTS

Ferredoxin	Absorbance of samples	Fe analysed (ppm)	E ($mM^{-1} \cdot cm^{-1}$)
Ferredoxin I	at 425 nm		at 425 nm
	0.118	1.13	11.639
	0.158	1.55	11.384
			11.512
Ferredoxin II	at 422 nm		at 422 nm
	0.102	1.02	11.168
	0.136	1.37	11.086
			11.127

The molecular weights of ferredoxins I and II were estimated to be about 18 000 by gel filtration on a Sephadex G-75 column. Both ferredoxins were identical in size and considerably larger than plant-type ferredoxins already studied.

Biochemical activities

The photosynthetic activity of *Nostoc* ferredoxins was investigated in the NADP photoreduction system, by using broken spinach chloroplasts. Ferredoxin II, which resembles spinach ferredoxin, had a higher activity than ferredoxin I at all ranges of ferredoxin concentration tested. High concentrations of *Nostoc* ferredoxins inhibited the NADP photoreduction. Spinach ferredoxin showed activity approximately equal to the average of the two ferredoxins.

Nostoc ferredoxins also mediated electron transfer in NADPH-cytochrome *c* reducing system, in which ferredoxin-NADP reductase from the same *Nostoc* cells was used. In this system, ferredoxin I had a higher activity than ferredoxin II in contrast with NADP photoreduction activity. They were also able to constitute the NADPH-cytochrome *c* reducing system with spinach ferredoxin-NADP reductase. These results suggest that prokaryotic *Nostoc* cells have a ferredoxin-NADP reductase system similar to that in eukaryotic higher plants. In this respect, a comparative study on this enzyme system might give some insight into the biochemical evolution of the photosynthetic electron transport system.

The experimental results in Table I show that the two ferredoxins were present in almost equal amounts. Concerning the physiological significance of the coexistence of the two ferredoxins in blue-green algae, this leads to the idea that ferredoxins are physiologically differentiated in the organism, because ferredoxin is involved not only in the photosynthetic electron transport system but also in the nitrogen-fixing system or other metabolic pathways in blue-green algae.

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