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Purification and characterization of ferredoxins from the heliobacterium *Heliobacillus mobilis*

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Introduction

Heliobacteria are anoxygenic phototrophic prokaryotes and have bacteriochlorophyll (BChl) *g* as the major photosynthetic pigment whose chemical structure is rather similar to chlorophyll (Chl) *a* than to BChl *a*. The molecular phylogeny of 16S rRNA sequence of eubacteria revealed that heliobacteria are related to the gram-positive bacteria. More recently, Xiong et al. (1998, 2000) suggested from their molecular phylogenetical studies that enzymes of heliobacteria which are involved in (B)Chl biosynthesis are the closest relatives to those of cyanobacteria among photosynthetic prokaryote. Thus it is very important to study the photosynthesis system of heliobacteria and compare it with those of other phototrophic organisms for elucidating evolution of photosynthesis. The reaction center (RC) of heliobacteria is considered to be similar to that of green sulfur bacteria and PSI of higher plants and cyanobacteria in that they contain very low-potential Fe-S cluster as the secondary electron acceptor. The electron transfer pathway around the RC in heliobacteria remains uncertain. To study the electron transfer pathway in heliobacteria from RC to ferredoxin (Fd) and further to electron acceptors, we have isolated and purified Fds from the heliobacterium *Heliobacillus mobilis*. Two Fds, termed Fd-A and Fd-B, were isolated from *H. mobilis* cells, and partially purified by ammonium sulfate fractionation, DEAE and Phenyl-Superose column chromatographies under anaerobic conditions.

Materials and methods

Cells of *H. mobilis* (ATCC43427) were grown anaerobically in 500 ml glass bottles for 18h at 37^{o}C, harvested by centrifugation under anaerobic conditions and stored at -80^{o}C until use. Fds were prepared as described basically in Seo et al. (2001) except that all operations were carried out under strictly anaerobic conditions. The frozen cells were suspended in a buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM sodium ascorbate, 0.5 mM sodium dithionite, 1 mM phenylmethanesulfonyl fluoride, 1 mM *p*-aminobenzamidine-HCl, 1 mM 6-amino-*n*-caproic acid, and 5 units/ml DNase, and disrupted by passing twice through a French pressure cell. After removing unbroken cells by centrifugation, the dark green supernatant was further centrifuged at 160,000 x *g* for 60 min. Powdered ammonium sulfate was added to the supernatant to 40% saturation, and the solution was gently stirred for 2 h at 4^{o}C. The mixture was centrifuged at 20,000 x *g* for 20 min, and ammonium sulfate was added to the

supernatant to 80% saturation. After gently stirring for overnight, a light brown precipitate was collected by centrifugation at 20,000 x *g* for 20 min and the precipitate was suspended in a buffer containing 50 mM Tris-HCl (pH 7.8) and dialyzed against the same buffer. The sample was diluted twofold with the buffer and then applied to a DEAE-cellulose column (2 cm x 12 cm, DE23, Whatman) which had been equilibrated with 50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 100 mM NaCl. After washing the column with a buffer containing 50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 200 mM NaCl, the Fds were eluted with a buffer containing 50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 500 mM NaCl. The brown colored fractions were collected and applied to a gel-permeation column (1 cm x 80 cm, Sephadex G-50, Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.8), 300 mM NaCl and eluted with the same buffer. The combined Fd-rich fractions were mixed with an equal volume of saturated ammonium sulfate solution in 50 mM Tris-HCl buffer (pH 7.8), and applied to a hydrophobic column (Phenyl Superose 10/10, Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.8) containing 2 M ammonium sulfate at room temperature. The column was washed with two column volumes of the medium for equilibration, and the Fds were eluted as two major peaks (385nm) with a 60 ml inverse linear gradient of ammonium sulfate of 2 to 0.8 M in 50mM Tris-HCl (pH 7.8). Each peak fraction was pooled separately, desalted with Ultrafree-4 (Millipore) and applied to an anion-exchange column (Mono Q 5/5, Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.8) containing 100 mM NaCl at room temperature. After washing the column with the same buffer, Fds were eluted with a 20 ml linear gradient of NaCl from 100 mM to 600 mM in the same Tris-HCl buffer. The purified Fds were stored either at 4^{o}C or at –80^{o}C until use. Concentrations of *H. mobilis* Fds were estimated from A_{385} assuming $\varepsilon_{385} = 30 \times 10^3$ M^{-1} cm^{-1} of *Chlorobium thiosulfatophilum* Fd (Hase et al. 1978). EPR measurements were performed using an EPR spectrometer (JES-RE2X, JEOL), equipped with a liquid helium cryostat. Absorption spectra were measured by a spectrophotometer, UV-3100PC (Shimadzu).

Results

Fds were precipitated by ammonium sulfate between 40-80% saturation and the precipitated fraction was further fractionated by DEAE-cellulose column chromatography. Fig. 1 shows absorption spectrum of brown colored fraction eluted from the DEAE column with a buffer containing 500 mM NaCl. Crude Fds sample

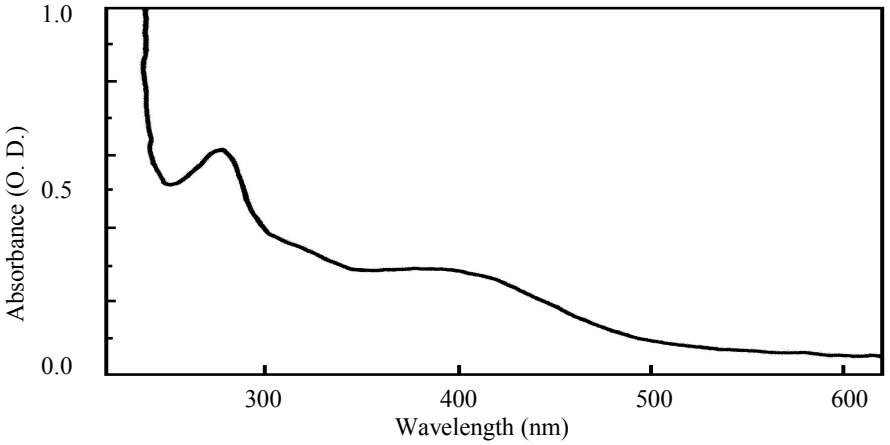

Fig. 1 Absorption spectrum of partially purified ferredoxins

Brown colored fractions were eluted from a DEAE column with a buffer containing 50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 500 mM NaCl. These fractions were combined and the absorption spectrum was measured.

was further purified with a Sephadex G-50 column and subsequently with a Phenyl Superose column. When Fds monitored at A_{385} were eluted from a Phenyl Superose column with a decreasing concentration gradient of ammonium sulfate, they eluted in two discernible peaks (A, B). The peak fractions A and B were separately pooled and purified by Mono Q ion-exchange column chromatography yielding Fd-A and Fd-B, respectively. The absorption spectra of Fd-A and Fd-B have absorption peaks at about 385 and 280 nm, with a shoulder at about 305 nm and trough at about 255 nm (data not shown). The *A*385/*A*280 ratios of the purified Fd-A and Fd-B were 0.73 and 0.75 respectively. Table 1 shows oxygen susceptibility of Fds. A_{385} of both Fds were unchanged for at least 8 h at 4^{o}C in anaerobic conditions. When air was bubbled to Fd-containing solutions, A_{385} of Fd-A were unchanged for 20 h at 4^{o}C, while, A_{385} of Fd-B were decreased to about a half in 2 h at 4^{o}C.

Table 1 Oxygen susceptibility of purified ferredoxins

	incubation time	
	0 h	2 h
Fd-A	0.25	0.25
Fd-B	0.30	0.15

Purified Fds were incubated at 4^{o}C under aerobic conditions and their *A*385 were measured.

Fig. 2 shows EPR spectra of Fds at cryogenic temperatures. No significant EPR signal was detected when oxidized Fd samples were incubated in the 50 mM Tris-HCl (pH 7.8). When 5 mM dithionite was added to the samples, Fd-A and Fd-B showed EPR signals at g = 2.05 and 1.94 and at g = 2.05 and 1.92, respectively.

Discussion

Seo et al. (2001) reported that at least three kinds of 2[4Fe-4S] are present in the green sulfur bacterium *Chlorobium tepidum.* The absorption spectra of Fd-A and Fd-B had peaks at 385nm and 280nm, and a shoulder at 300 nm, which were very similar to a typical absorption spectrum of 2[4Fe-4S] type Fds from various bacteria. These Fds were stable against oxygen. With heliobacterial Fds, although Fd-A is very stable against oxygen, Fd-B is very susceptible to oxygen, indicating a possibility that the functions of Fd-A and Fd-B in heliobacteria differ. Cryogenic EPR measurements indicated Fd-A had EPR signals around at g= 2.05, 1.94 and Fd-B around at g= 2.05, 1.92, respectively. Both Fds were fully reduced by dithionite at pH 8.0.

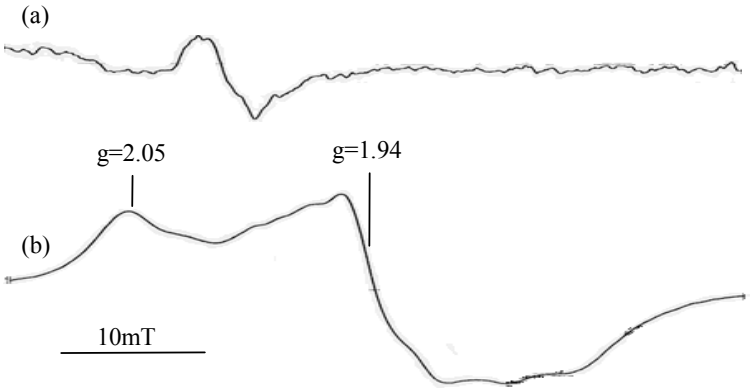

Fig. 2 EPR spectra of Fd-A

(a) Fd-A (35 μM) was suspended in a medium containing 50 mM Tris-HCl (pH 7.8) and frozen in liquid nitrogen. (b) After recording spectrum (a), the sample was thawed and 5 mM dithionite was added to the mixture.
EPR conditions: temperature, 15K; microwave power, 20 mW; frequency 9.07 GHz; modulation amplitude 1 mT.

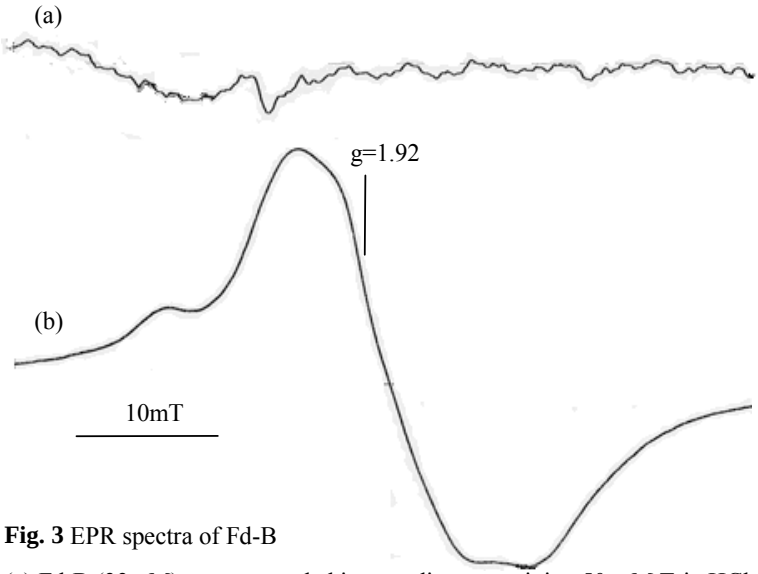

Fig. 3 EPR spectra of Fd-B

(a) Fd-B (33 μM) was suspended in a medium containing 50 mM Tris-HCl (pH 7.8) and frozen in liquid nitrogen. (b) After recording spectrum (a), the sample was thawed and 5 mM dithionite was added to the mixture.
EPR conditions: temperature, 15K; microwave power, 20 mW; frequency 9.07 GHz; modulation amplitude 1 mT.

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