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Purification and characterization of a ferredoxin from *Haloarcula japonica* strain TR-1

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Abstract

A ferredoxin (Fd) was purified from the extremely halophilic archaeon, *Haloarcula japonica* strain TR- 1, to electrophoretic homogeneity. The apparent molecular weight (M_r) of the Fd was estimated to be 24,000 on SDS-polyacrylamide gel electrophoresis. The amino acid composition analysis revealed that the Fd composed of a number of acidic amino acids (uncorrected for amides). The *N*-terminal amino acid sequence (30 residues) was determined to be: PTVEYLNYEVVDDNGWDMYDDDVFAEASDM. The iron content was 3.42 ± 0.04 mol/mol-Fd on the basis of the apparent M_r value. The absorption and ESR spectra of the Fd showed similarity to those of Fds from plant and *Halobacterium halobium*. These results led us to conclude that the *H. japonica* Fd contained a [2Fe-2S] cluster.

Abbreviations: Fd – ferredoxin; iron-sulfur, Fe-S – extremely halophilic archaea, halobacteria.

Introduction

Ferredoxins (Fds) are small iron-sulfur (Fe-S) proteins containing one or two Fe-S clusters, and are supposed to act as electron carrier. As Fe-S clusters, [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters have been found in Fds from many organisms. To date, [2Fe-2S] Fds have been isolated from plants, algae, and cyanobacteria; others mainly from eubacteria (Hall *et al.* 1974; Thomson 1985).

Extremely halophilic archaea (halobacteria) are extremophiles which grow in hypersaline environments, such as the Dead Sea, the Great Salt Lake, and a salt farm. These halobacteria are currently divided into ten genera, *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halorubrum*, *Halobaculum*, *Natrialba*, *Natronomonas*, *Natronobacterium*, and *Natronococcus* (Grant & Larsen 1989; Kamekura *et al.* 1997).

Halobacterial Fds from *H. halobium* (Kerscher *et al.* 1976) and *Halobacterium* of the Dead Sea (Werber & Mevarech 1978b) [identified as *Haloarcula marismortui* (Werber & Mevarech 1978a)] have been purified and characterized. These Fds contain a [2Fe-2S] cluster in the protein molecule. Some of their properties are known to be similar to those of plant and algal Fds. It has been reported that halobacterial Fds can also work as electron carriers *in vivo* (Kerscher & Oesterhelt 1977; Werber & Mevarech 1978a). Recently, Frolow *et al.* (1996) reported about protein adaptation of *H. marismortui* Fd to a high salt environment.

Haloarcula japonica strain TR-1, isolated from a saltern soil located at Noto Peninsula in Japan, is a predominantly triangular disc-shaped extremely

halophilic archaeon (Takashina *et al.* 1990). Taxonomic and morphological characteristics have been studied for the strain to date [for review see Horikoshi *et al.* (1993)]. In this paper, we report the purification and characterization of a Fd from *H. japonica*.

Materials and methods

Strain, medium and culture conditions

H. japonica strain TR-1 (ATCC 49778) was cultivated in a 2 l buffle-walled Erlenmeyer flask containing a 400 ml of the *H. japonica* medium (Nishiyama *et al.* 1995) at 37 °C under aerobic conditions as described previously (Nakamura *et al.* 1992). The cells were harvested by centrifugation at $7000 \times g$ for 10 min at 4 °C, and then stored at −80 °C. Five to ten grams of cell paste/liter of culture medium was obtained.

Purification of Fd

Thirty grams of cell paste were suspended in 90 ml of the salt solution [20% (w/v) NaCl and 4% (w/v) $MgSO_4 \cdot 7H_2O$]. The cell suspension was sonicated on ice at 100 W output for a total of 10 min (20 times of 30 s pulses and appropriate rest intervals to cool the suspension below 4 °C) with a TOMY (Tokyo, Japan) UD-201 sonicator. The sonicated suspension was centrifuged at $25,800 \times g$ for 20 min. The supernatant was further centrifuged at $203,000 \times g$ for 1 h. The following procedures were performed at room temperature. To desalt, obtained supernatant (cell-free extract, 98 ml) was applied to a Sephadex G-25 (Pharmacia; Uppsala, Sweden) column (4.6×30 cm), equilibrated with 0.15 M Tris/HCl (pH 7.0) at a linear flow rate of 29 cm/h. The Fd-containing red-colored fractions were pooled. The reactions collected were loaded onto a DEAE Sepharose Fast Flow (Pharmacia) column (2.5×33 cm), equilibrated with the same buffer at a linear flow rate of 37 cm h^{-1}. The adsorbed proteins were eluted with 600 ml of a linear gradient from 0 to 1 M NaCl in the same buffer at a linear flow rate of 18 cm h^{-1}. The Fd started to elute at 0.35 M NaCl. The Fd-containing fractions (absorbance based on the cluster: $A_{420} > 0.21$) were collected and concentrated to 6 ml by ultrafiltration using an Amicon (Beverly, USA) Diaflo with a PM10 membrane. The concentrated proteins were applied to a Sephacryl S-200 HR (Pharmacia) column (2.5×85.5 cm) equilibrated with 0.15 M Tris/HCl (pH 7.0) containing 0.1 M NaCl. The column was run at a linear flow rate of 9.8 cm h^{-1}. The fractions containing Fd (purity index: $A_{420}/A_{280} > 0.14$) were pooled and loaded onto a DEAE Sepharose Fast Flow column (1.0×9.5 cm) equilibrated with the same buffer at a linear flow rate of 153 cm h^{-1}. The adsorbed proteins were eluted with 80 ml of a linear gradient from 0 to 1 M NaCl in the same buffer at a linear flow rate of 38 cm h^{-1}. The Fd started to elute at 0.3 M NaCl. The Fd fractions ($A_{420}/A_{280} > 0.24$) were pooled and stored in a vial under nitrogen at 4 °C.

SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done on a 15% (w/v) polyacrylamide gel by the method of Laemmli (1970). The low range molecular weight standards (Bio-Rad; Richmond, USA) were used as molecular mass markers. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (CBB).

Protein analyses

Protein concentrations were routinely estimated by the modified method (Bensadoun & Weinstein 1976) of Lowry *et al.* (1951) with bovine serum albumin, fraction V (Sigma; St. Louis, USA) as the standard. The iron content of the Fd were measured by ICP plasma emission spectroscopy using a Seiko Instruments (Chiba, Japan) SPS 1500VR plasma spectrometer. The amino acid composition was estimated with a Hitachi (Tokyo, Japan) L-8500 automatic amino acid analyzer. Samples were hydrolyzed with 6 M HCl for 24 h at 110 °C. The *N*-terminal amino acid sequence of the Fd was determined by automated Edman degradation of the native protein with a Shimadzu (Kyoto, Japan) PPSQ-10 protein sequencer and an on-line PTH-10 PTH analyzer.

Spectroscopy

Absorption spectra were recorded on a Hitachi U-2000 spectrophotometer and ESR spectra on a JEOL (Tokyo, Japan) JES-RE3X spectrometer interfaced to an ESPRIT 375 ESR Data System and equipped with a Liquid transfer HELI-TRAN model LTR-3 (APD Cryogenics; Allentown, USA). Absorption and ESR spectra were measured in 0.15 M Tris/HCl (pH 7.0) containing 0.33 NaCl and 3.1 M KCl. The samples were degassed under vacuum, replaced with argon gas, and then reduced by adding an oxygen-free solution of sodium dithionite.

Table 1. Purification of the *H. japonica* Fd.

Purification step	Total protein (mg)	A_{420}/A_{280} [a]	Purification factor (fold)
Cell-free extract	1700	0.04	1
Sephadex G-25	1440	0.04	1
1st DEAE Sepharose	183	0.02	0.5
Sephacryl S-200	17.2	0.22	5.5
2nd DEAE Sepharose	12.8	0.29	7.3

[a]Purity index for a [2Fe-2S] Fd.

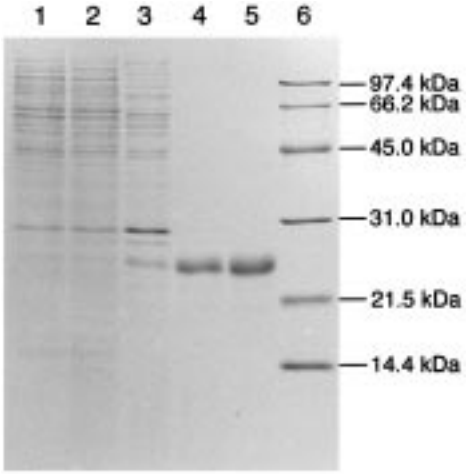

Fig. 1. SDS-PAGE of the *H. japonica* Fd during purification. A 15% (w/v) polyacrylamide gel was used. After electrophoresis, proteins in the gel were stained with CBB. Lane 1, cell-free extract; lane 2, fraction from the Sephadex G-25 column; lane 3, fraction from the 1st DEAE Sepharose column; lane 4, fraction from the Sephacryl S-200 column; lane 5, fraction from 2nd DEAE Sepharose column; lane 6, molecular mass markers. Each lane contained 1.55 μg of proteins.

Results and discussion

Purification of the H. japonica *Fd*

Purification steps were monitored by SDS-PAGE (Figure 1). Results of a typical purification are summarized in Table 1. The *H. japonica* Fd was purified to electrophoretic homogeneity, and the purity index (A_{420}/A_{280}) of the final preparation was 0.29. The purification factor was estimated about 7.3-fold based on the purity index values. The drop in purity index on the first DEAE Sepharose would be caused because contaminants exhibited absorption at 420 nm were eliminated by this step. Because of such contaminants, the A_{420}/A_{280} value of the cell-free extract would essentially be lower. Thus, the purification fold of the Fd isolated would be higher than 7.3 in practice. The purity index value of the purified *H. japonica* Fd was almost same as those of other halobacterial Fds (Kerscher *et al.* 1976; Werber & Mevarech 1978b). About 13 mg of the purified Fd was obtained from 30 g (wet-weight) of the cells containing 1700 mg of soluble proteins, suggesting that the Fd content in *H. japonica* was about 1%.

Molecular properties of the H. japonica *Fd*

The purified Fd gave a single protein band on SDS-PAGE (Figure 1, lane 5), and this band corresponded to an apparent molecular weight (M_r) of 24,000. Table 2 shows the amino acid composition of the *H. japonica* Fd, as well as those of Fds from other organisms. Since the samples were hydrolysed with HCl, Trp and Cys were not exactly determined. We found that the *H. japonica* Fd, as well as other halobacterial Fds, is also composed of many Asx and Glx. In analogy with other halobacterial Fds which comprise a number of Asp and Glu, the *H. japonica* Fd will also contain many Asp and Glu. Higher contents of acidic amino acid residues have been found in many other halobacterial proteins (Lanyi 1974). From these results, we consider that since the *H. japonica* Fd would contain many acidic amino acids, on SDS-PAGE the M_r is overestimated; on gel filtration the observed M_r is likely influenced by the protein structure and the folding. Figure 2 shows the result of the *N*-terminal amino acid sequence analysis. The *N*-terminal sequence (30 residues) was determined to be: PTVEYLNYEVVDDNGWDMYDDDVFAEASDM. We found that the *N*-terminal sequence of the *H. japonica* Fd has 96.7% identity with the *H. marismortui* Fd (Hase *et al.* 1980) and 70.0% with the *H. halobium* Fd (Hase *et al.* 1978). This result corresponds to the taxonomic distances among *H. japonica*, *H. marismortui* and *H. halobium*.

Table 2. Amino acid compositions of ferredoxins from *H. japonica* and other organisms.

Amino acid	*H. japonica*[a]	*H. halobium*[b]	*H. marismortui*[c]	Spinach[d]	*E. coli*[e]
			(mol%)		
Lys	2.45	4.03	2.43	4.12	3.82
His	1.08	0.712	0.81	1.03	5.02
Arg	2.49	2.27	2.32	1.03	5.97
Cys	0.836	3.43	3.33	5.15	N.D.[f]
Asx	21.0	16.9	21.8	13.4	10.9
Thr	1.63	3.32	1.87	8.25	5.02
Ser	3.96	3.31	3.73	7.22	5.85
Glx	14.9	16.9	13.9	13.4	18.0
Pro	1.91	2.27	2.36	4.12	6.09
Gly	9.81	7.72	6.85	6.19	5.97
Ala	10.4	11.5	8.76	9.28	9.55
Val	7.65	5.54	7.51	7.22	4.18
Met	3.22	3.03	3.00	0.00	1.02
lle	4.47	4.88	5.11	4.12	6.33
Leu	7.93	7.32	7.00	8.25	10.3
Tyr	5.30	5.49	6.19	4.12	1.19
Phe	0.982	1.52	1.61	2.06	0.824
Trp	N.D.[f]	N.D.[f]	1.43	1.03	N.D.[f]

[a]This work; [b]ref. period, (Hase *et al.* 1978); [c]ref. (Hase *et al.* 1980); [d]ref. (Werber & Mevarech 1978b); [e]ref. (Knoell & Knappe 1974); [f]not determined.

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                1         10          20          30
H. marismortui a PTVEY LNYEV VDDNG WDMYD DDVFA EASDM
                ***** ***** ***** ***** ****  *****
H. japonica    b PTVEY LNYEV VDDNG WDMYD DDVFG EASDM
                ***** ****   ** * *** * ** *   * *
H. halobium    c PTVEY LNYET LDDQG WDMDD DDLFE KAADA
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Fig. 2. N-terminal amino acid sequence of Fds from *H. japonica* and other halobacteria. Asterisks indicate the identical residues. [a]ref. (Hase *et al.*, 1980); [b]this work; [c]ref. (Hase *et al.* 1978).

The iron content of the *H. japonica* Fd was measured and calculated to be 3.42 ± 0.04 mol/mol-Fd based on the observed protein concentration and the apparent M_r of 24,000. Since the *H. japonica* Fd consists of many acidic amino acid residues, it is possible that the apparent M_r determined by SDS-PAGE is overestimated. This interpretation is consistent with the preliminary mass spectroscopic observation: M_r of the *H. japonica* Fd was determined to be about 15,000 on matrix assisted laser desorption mass spectrometry. The iron content of the *H. japonica* Fd can be evaluated to be 2.1 mol/mol-Fd based on the M_r of 15,000, suggesting that the *H. japonica* Fd contains a [2Fe-2S] cluster per molecule. It has been reported that the M_r of Fds from *H. halobium* and *H. marismortui* also tend to be overestimated on SDS-PAGE (Kerscher *et al.* 1976; Werber & Mevarech 1978b). The *H. japonica* Fd eluted as a single peak after gel filtration corresponding to M_r of 27,900 (data not shown). Thus, the *H. japonica* Fd might take a dimeric structure in an aqueous solution.

Spectroscopic properties of the H. japonica *Fd*

Halobacteria are known to contain very high concentrations of K^+ (about 3 M), instead of Na^+, in their cytoplasm (Lanyi 1974; Meseguer *et al.* 1995). Therefore, absorption and ESR spectra were measured in the buffer containing a high concentration of KCl (3.1 M).

The absorption spectra of the oxidized and reduced Fds are shown in Figure 3. The absorption maxima of the oxidized Fd are at 275, 329, 420, and 465 nm. On reduction with sodium dithionite, the absorption decreased by 53.0% at 420 nm and 50.2% at 465 nm. The shape of absorption spectrum and the positions of maxima were similar to those of halobacterial (Kerscher *et al.* 1976; Werber & Mevarech 1978b), spinach (Tagawa & Arnon 1962; Palmer *et al.* 1967; Hall *et al.* 1974) and adrenodoxin-type Fds (Palmer *et al.* 1967; Knoell & Knappe 1974). The absorption spectra were measured in the same buffer without KCl or the buffer containing 1.5 M KCl. The spectrum patterns were almost identical to those measured under higher-KCl conditions (data not shown).

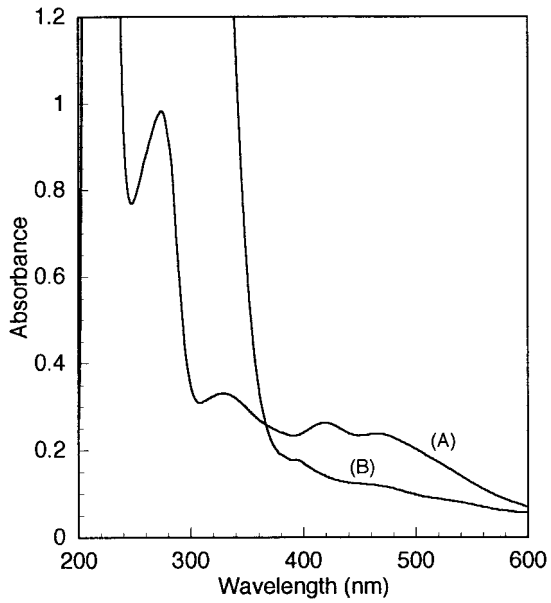

Fig. 3. Absorption spectra of the *H. japonica* Fd. (A) The oxidized Fd (0.343 mg ml^{-1}) in 0.15 M Tris/HCl buffer (pH 7.0) containing 0.33 M NaCl and 3.1 M KCl. (B) The same sample reduced with sodium dithionite.

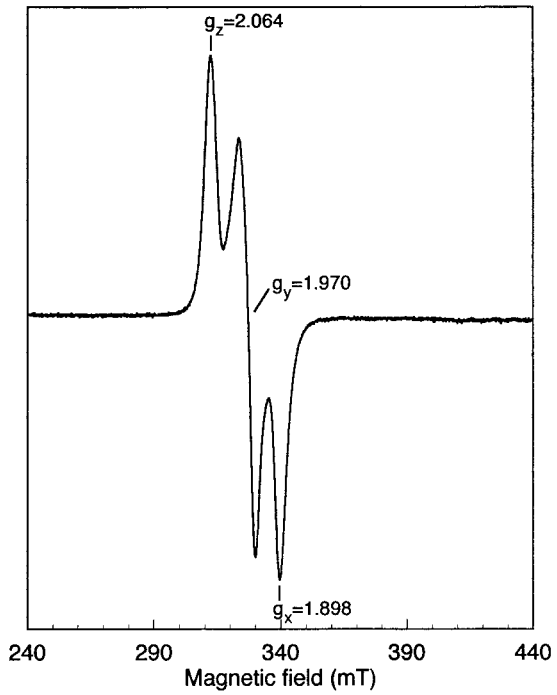

Fig. 4. ESR spectrum of the dithionite-reduced *H. japonica* Fd. The Fd [3.19 mg ml^{-1}, in 0.15 M Tris/HCl buffer (pH 7.0), 0.33 M NaCl, 3.1 M KCl] was reduced with sodium dithionite. The spectrum was recorded at 25.5±1 K by using 1 mW of microwave power. The spectrometer settings were as follows: gain, 63; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; time constant, 30 ms; sweep time, 240 s; microwave frequency, 9.02 GHz.

The ESR spectrum of the dithionite-reduced *H. japonica* Fd recorded near 25 K is shown in Figure 4. The spectrum was rhombic with three g-values $g_x = 1.898$, $g_y = 1.970$, $g_z = 2.064$ and the average g-value 1.977. The signals were similar to those of Fds from *H. halobium* (Kerscher *et al.* 1976) and spinach (Hall *et al.* 1974). Furthermore, almost the same ESR spectra were obtained in the lower-KCl buffers (0 M and 1.5 M KCl, data not shown). No signals were detected with the oxidized Fd.

The absorption and ESR spectra of the *H. japonica* Fd clearly indicated that the Fd had a similar chromophore to other halobacterial and plant Fds. Consequently, we concluded that the *H. japonica* Fd contained the [2Fe-2S] cluster in the molecule. Considering the iron content described above, we propose that the *H. japonica* Fd has a [2Fe-2S] cluster per molecule.

Most halobacterial enzymes are denatured and inactivated at NaCl (or KCl) concentrations below 1 M. It has been reported that malate dehydrogenase from *H. marismortui* is renatured and reactivated after 2 h of exposure to high concentrations of salt (Mevarech & Neumann 1977). On the other hand, the changes in the salt concentration had no effect on the absorption and ESR spectra of the *H. japonica* Fd. Therefore, the protein structure, especially near the [2Fe-2S] cluster, of the *H. japonica* Fd was not influenced by the ambient salt concentrations.

The Fd was present in large amounts (ca. 1% of the total soluble protein) in *H. japonica*, as well as in two other halobacteria, *H. marismortui* and *H. halobium* (Kerscher *et al.* 1976; Werber & Mevarech 1978b). And Rouault and Klausner (1996) reviewed about the function of Fe-S protein *in vivo*. However, physiological roles of the Fd in *H. japonica* are not clear yet. Further studies are in progress to reveal the functions of the *H. japonica* Fd.

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