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メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	https://doi.org/10.24517/00011007

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Purification and Characterization of a [3Fe-4S] [4Fe-4S] Type Ferredoxin from Hyperthermophilic Archaeon, *Pyrobaculum islandicum*¹

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Received for publication, October 30, 1997

A ferredoxin was purified from the hyperthermophilic archaeon, *Pyrobaculum islandicum*. EPR spectra and metal content analyses suggested that the ferredoxin molecule contained one [3Fe-4S] and one [4Fe-4S] cluster. The ferredoxin was rapidly reduced by 2-oxoglutarate:ferredoxin oxidoreductase purified from *P. islandicum*, indicating that it functions physiologically as an electron sink for the redox enzymes participating in glycolytic metabolism. Furthermore, the amino acid sequence of the *P. islandicum* ferredoxin was compared with those of several other bacterial ferredoxins.

Key words: ferredoxin, hyperthermophile, iron-sulfur cluster, *Pyrobaculum islandicum*.

The hyperthermophilic archaeon, *Pyrococcus furiosus*, has many kinds of ferredoxin-related enzymes, such as aldehyde ferredoxin oxidoreductase (1), formaldehyde ferredoxin oxidoreductase (2, 3), glyceraldehyde-3-phosphate ferredoxin oxidoreductase (4), sulfide dehydrogenase, and sulfhydrogenase (5-8), indicating that ferredoxin essentially participates in various metabolic pathways. On the other hand, the ferredoxin from the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7, serves as an intermediate electron transfer protein between coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductase and a novel iron-sulfur flavoprotein (9, 10). Recently, Fujii *et al.* determined the 2.0 Å resolution crystal structure of the ferredoxin from *Sulfolobus* sp. strain 7 (11, 12), and showed that a zinc atom is tightly coordinated by three histidine residues in the N-terminal extension region and one aspartate residue in the core fold. Furthermore, Iwasaki *et al.* have suggested that the consensus sequence is ligand to the novel zinc center in the N-terminal extension region that is characteristic of thermoacidophilic archaeons (13).

As described above, ferredoxin is thought to be a key electron transfer protein in Archea. On the other hand, the hyperthermophilic archaeon, *Pyrobaculum islandicum*, is a facultative chemoautotrophic archaeon and produces ATP through anaerobic respiration (14). Recently, Seilig and Schönheit reported that all of the enzymatic activities involved in the citric acid cycle are present in *P. islandicum*

(15). Furthermore, the archaeon can utilize thiosulfate and elemental sulfur as terminal electron acceptors, and thereby produce CO₂ and H₂S under heterotrophic growth conditions (16). Therefore, the archaeon seems to have metabolic pathways for oxidizing organic compounds to CO₂ in the presence of elemental sulfur or thiosulfate, suggesting that ferredoxin plays an essential role as an electron mediator between acetyl-CoA oxidation and elemental sulfur reduction or thiosulfate reduction, much like the role of the ferredoxin of *P. furiosus* in glucose fermentation.

In the present study, we first purified ferredoxin from the archaeon in an electrophoretically homogeneous state and investigated its molecular properties. We then determined its amino acid sequence and compared it with those of several other bacterial type ferredoxins.

MATERIALS AND METHODS

Bacterium and Growth Conditions—*P. islandicum* (DSM 4184) was cultivated anaerobically at 95°C for 2 days in a chemically defined medium (16) with slight modifications. Sodium thiosulfate was added to the medium as an electron acceptor. The cells were harvested by centrifugation at 10,000 × *g* for 15 min and then stored at -80°C until use.

Physical Measurements—Spectrophotometric measurements were performed with a Shimadzu MPS-2000 spectrophotometer and a Hitachi 220A with 1-cm light path cuvettes. The metal content was determined with an inductively coupled Plasma Spectrometer SPS 1500 VR (Seiko Instruments, Tokyo) after the sample had been dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA for 18 h. Electron paramagnetic resonance measurements were carried out with a Bruker ESP-300E spectrometer or a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system. MALDI-TOF mass spectrometry was performed

¹This work was supported by a Grant-in-Aid for Molecular Biometallics (No. 09235207) to Y.F. from the Ministry of Education, Science and Culture of Japan.

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Abbreviations: CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; CM-Fd, carboxymethylated ferredoxin; PVDF, polyvinylidene difluoride; TFA, trifluoro acetic acid.

with a PerSeptive Biosystems Voyager RP instrument at an accelerating potential of 25 kV, using 3,5-dimethoxy-4-hydroxycinnamic acid.

SDS-PAGE was performed by the method of Schägger and von Jacow (17). The sample for SDS-PAGE was denatured by heating at 100°C with 1% (w/v) SDS and 1% (v/v) β -mercaptoethanol for 30 min. The molecular weight of the native protein was determined by gel filtration on a Sephadex G-75 column (2.4 \times 115 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl. The column was calibrated with the following standard proteins: trypsin inhibitor (M_r = 21,500), horse heart cytochrome *c* (M_r = 12,375), and lysozyme (M_r = 14,300) [lysozyme behaves as a protein species with a molecular weight of 5,000 (18)].

The protein content was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as a standard.

Partial Purification of 2-Oxoglutarate Ferredoxin Oxidoreductase from *P. islandicum*—2-Oxoglutarate:ferredoxin oxidoreductase was partially purified from *P. islandicum* under anaerobic conditions because of its oxygen sensitivity. Frozen cells were suspended in 10 mM Tris-HCl buffer, pH 8.0, which had been degassed and flushed with pure N₂, and then disrupted at 4°C in a French pressure cell at 1,100 kg/cm². After the unbroken cells had been removed by centrifugation at 10,000 $\times g$ for 15 min, the supernatant obtained was degassed and flushed with N₂, and further centrifuged at 104,000 $\times g$ for 1 h. The supernatant, which had been degassed and flushed with N₂, was charged onto a DEAE-Toyopearl column (2.6 \times 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, in the presence of N₂. After the column had been washed with 200 ml of the same buffer, 2-oxoglutarate:ferredoxin oxidoreductase was eluted with a linear gradient produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0, and the buffer containing 0.4 M NaCl. The eluate containing 2-oxoglutarate:ferredoxin oxidoreductase activity was concentrated by ultrafiltration in a Amicon unit (Centriflo CF25) and then stored under N₂ gas at -80°C.

Enzyme Assay—*P. islandicum* 2-oxoglutarate:ferredoxin oxidoreductase activity was measured at 70°C by the method of Zhang *et al.* (10), using *P. islandicum* ferredoxin as an intermediate electron acceptor. The reaction mixture comprised 50 mM potassium phosphate buffer, pH 7.5, 2 mM 2-oxoglutarate, 50 μ M CoA, 18.8 μ M horse cytochrome *c*, and 0.3 μ M *P. islandicum* ferredoxin. The reaction was started by addition of the enzyme to the reaction mixture. Although the reduction of horse cytochrome *c* was followed by measuring the increase in the absorbance at 550 nm, the activity could not be measured at over 70°C, because of denaturation of the horse cytochrome *c* at high temperature.

Determination of the Amino Acid Sequence—The chemical digestion of ferredoxin, which was carboxymethylated according to Crestfield *et al.* (19), was performed with formic acid as follows: about 130 μ g CM-Fd was incubated in 2% (v/v) formic acid for 2 h at 108°C by the method of Inglis (20). Enzymatic digestions with trypsin or V8-protease was performed by treating about 130 μ g of CM-Fd with 10 μ g *Staphylococcus aureus* V8-protease or 10 μ g TPCK-trypsin, respectively, in 50 mM NH₄HCO₃ containing 2 mM EDTA for 24 h at 37°C. Enzymatic digestion with

proline-specific endopeptidase was performed by treating about 130 μ g of CM-Fd with 10 μ g proline-specific endopeptidase in 50 mM sodium phosphate buffer, pH 7.0, for 4 h at 37°C. The digested ferredoxins were lyophilized and then fractionated by reverse-phase HPLC on a Cosmosil 5C18 column (4.6 \times 250 mm) in 0.1% (v/v) trifluoroacetic acid (TFA) with a linear gradient of 0 to 50% (v/v) acetonitrile. The peptides in the eluate were monitored as to the absorbance at 230 nm.

The deblocking of the modified N-terminal amino acid residues such as *N*-acetylserine or *N*-acetylthreonine was performed by the method described by Wellner *et al.* (21) with some modifications, the ferredoxin in the SDS-PAGE gel was transferred to the PVDF membrane filter at room temperature by the method of Towbin *et al.* (22), using 0.5% (w/v) SDS. After the filter had been stained with Coomassie Brilliant Blue R-250, the ferredoxin band was cut down and incubated in gas phase anhydrous trifluoroacetic acid (TFA) for 1 h at 60°C. To deblock pyroglutamate, *P. furiosus* pyroglutamate aminopeptidase was used.

The amino acid sequence of ferredoxin was determined with a gas-phase protein sequencer (Applied Biosystems, model 470A) equipped with an on-line PTH-analyzer (Applied Biosystems, model 120A, USA), and a gas-phase protein sequencer (Shimadzu, PPSQ-21, Tokyo) equipped with a UV-VIS Detector (Shimadzu, SPD-10A) and a liquid chromatograph (LC-10AS).

Phylogenetic Tree Analysis—Phylogenetic trees were constructed by means of the parsimony and neighbor-joining methods using the sequence interpretation tool, CLUSTALW (<http://www.genome.ad.jp/SIT/SIT.html>). All amino acid sequence data for ferredoxins used for phylogenetic calculations in this study were obtained from the GenEMBL, PIR, and SWISS-PROT data banks, except those for the ferredoxin from *P. islandicum*.

Reagents—CM-Cellulose and Sephadex G-75 were purchased from Pharmacia Fine Chemicals (Sweden). DEAE-Toyopearl was purchased from Tosoh Corporation (Japan). The molecular weight markers for SDS-PAGE were purchased from Fluka AG (Switzerland). *P. furiosus* pyroglutamate aminopeptidase was purchased from Takara Biomedicals (Japan). Proline-specific endopeptidase was purchased from Seikagaku (Japan). TPCK-trypsin was purchased from Worthington Biochemical (USA). *S. aureus* V8-protease was purchased from Wako Pure Chemical (Japan).

RESULTS AND DISCUSSION

Purification of Ferredoxin from *P. islandicum*—Frozen cells (about 20 g wet weight) harvested from about 150 liter culture with sodium thiosulfate were suspended in 270 ml of 10 mM Tris-HCl buffer, pH 8.0, and then disrupted in a French pressure cell (1,100 kg/cm²). All subsequent steps were performed under aerobic conditions at room temperature. Unbroken cells were removed by centrifugation at 10,000 $\times g$ for 15 min. The resulting supernatant was used as a cell-free extract. The cell-free extract was further centrifuged at 104,000 $\times g$ for 1 h, and the supernatant obtained was charged onto a DEAE-Toyopearl column (2.6 \times 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. After the column had been washed with 200 ml of the same buffer, ferredoxin was eluted with a linear gradient

produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0, and the buffer containing 0.4 M NaCl. The eluates which contained ferredoxin were combined and concentrated by pressure filtration on an Amicon YM-3 membrane under N₂ gas at 4°C. The concentrated fraction was subjected to gel filtration on a Sephadex G-75 column (2.4×115 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. To the eluates which contained the ferredoxin, ammonium sulfate was added to give a final concentration of 2.06 M, and then the suspension was stirred for 30 min. After the resulting solution had been centrifuged at 10,000×g for 15 min, the supernatant was charged onto a CM-cellulose column (1.0×3 cm) equilibrated with the 10 mM Tris-HCl buffer, pH 8.0, containing 2.06 M ammonium sulfate. Ferredoxin was eluted with a linear gradient produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0, containing 2.06 M ammonium sulfate, and the buffer containing 1.35 M ammonium sulfate. The eluates were used as the purified ferredoxin preparation. About 0.83 mg of purified ferredoxin was obtained from about 20 g cells.

Characterization of the *P. islandicum* Ferredoxin—The molecular weight of the *P. islandicum* ferredoxin was estimated to be 11,500 by SDS-PAGE, while the molecular weight of the native *P. islandicum* ferredoxin was estimated to be 11,500 by gel filtration analysis (data not shown). Furthermore, MALDI-TOF mass spectrometry of the denatured apoprotein after heating at 100°C with 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol gave an average mass of 11,910±50. These results show that the *P. islandicum* ferredoxin exists in monomeric form in aqueous solution.

The metal contents of *P. islandicum* ferredoxin were determined with an inductively coupled Plasma Spectrometer. The ferredoxin contained 32.4 μg non-heme irons per mg protein, indicating that it has 7 iron atoms per mol of protein. Zn, Mo, Ni, Mn, and Cu were scarcely detected in the purified preparation.

Figure 1 shows the absorption spectra of *P. islandicum* ferredoxin. This ferredoxin showed an absorption peak at 282 nm and a broad peak at around 400 nm in the air-oxidized form (Fig.1A), and the spectra did not change on incubation for 2 h at 90°C. Upon the addition of excess solid

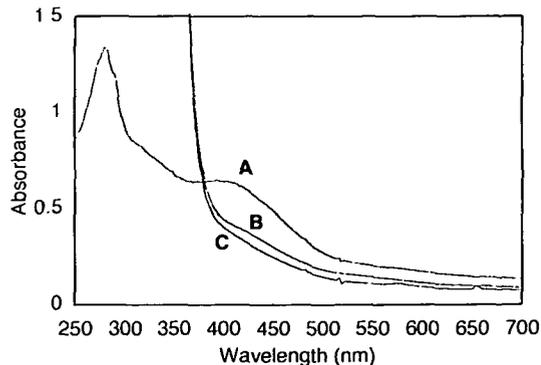


Fig. 1. Absorption spectra of *P. islandicum* ferredoxin. The ferredoxin (0.256 mg/ml) was dissolved in 20 mM sodium phosphate buffer, pH 7.0 (A, B), or 600 mM CAPS buffer, pH 9.3(C). A, air-oxidized form; B and C, reduced form prepared by the addition of excess amounts of Na₂S₂O₄.

Na₂S₂O₄ to the ferredoxin solution, the broad peak at around 400 nm decreased by 31% in 20 mM sodium phosphate buffer (pH 7.0) (Fig. 1B). Because further addition of Na₂S₂O₄ did not induce any spectral changes at around 400 nm, the ferredoxin seems to have been held in the partially-reduced state under the experimental conditions. However, when the ferredoxin was suspended in 600 mM CAPS buffer, pH 9.3, it was more highly reduced with Na₂S₂O₄

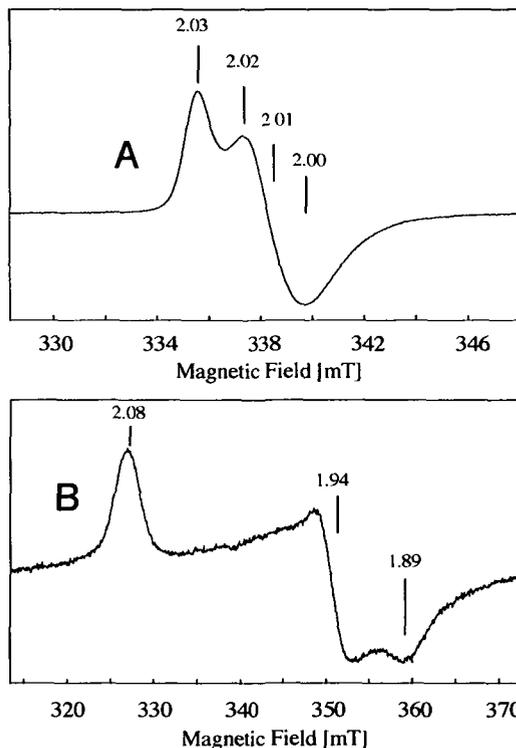


Fig. 2. Low temperature EPR spectra of *P. islandicum* ferredoxin in the air-oxidized (A) and dithionite-reduced (B) forms. The sample was dissolved in 600 mM CAPS buffer, pH 9.3. Instrument settings for EPR spectroscopy: temperature, 12.5 K (A), 12.7 K (B); microwave power, 0.51 mW (A), 1.0 mW (B); modulation amplitude, 0.31 mT; g values, as indicated.

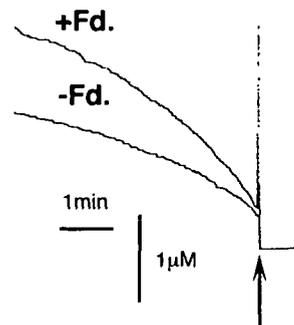


Fig. 3. Reconstitution of electron transfer between ferredoxin and 2-oxoglutarate with 2-oxoglutarate:ferredoxin oxidoreductase of *P. islandicum*. The reaction mixture comprised 50 mM potassium phosphate buffer, pH 7.5, 2 mM 2-oxoglutarate, 50 μM CoA, 18.8 μM horse cytochrome c, and 0.3 μM *P. islandicum* ferredoxin. The reduction of horse heart cytochrome c was spectrophotometrically followed at 70°C as described under "MATERIALS AND METHODS."

(Fig. 1C). Therefore, it seems likely that the *P. islandicum* ferredoxin has an [Fe-S] cluster with a pH-dependent and unusually low redox potential.

Figure 2 shows the EPR spectra of *P. islandicum* ferredoxin. When the ferredoxin was suspended in 600 mM CAPS buffer, pH 9.3, the oxidized form showed a sharp $g=2.03$ signal at 12.5K (Fig. 2A). The lineshape of the signal was, in general, similar to those observed for proteins containing an oxidized $[3\text{Fe-4S}]^{1+}$ center (23–26). A signal with $g=2.03$ was also observed for the oxidized form at pH 7.0. Reduction of the ferredoxin with excess solid $\text{Na}_2\text{S}_2\text{O}_4$ at pH 9.3 decreased the $g=2.03$ signal, and produced a broad rhombic EPR spectrum with $g=2.08$, 1.94 and 1.89 signals (Fig. 2B). These signals are similar to those of a reduced $[4\text{Fe-4S}]^{1+}$ cluster (26, 27). Therefore, it seems likely that the *P. islandicum* ferredoxin has a $[3\text{Fe-4S}]$ cluster and a $[4\text{Fe-4S}]$ cluster in the monomeric form.

Reconstitution of the Ferredoxin-Dependent Redox System of *P. islandicum*—Archaea such as *P. furiosus* and *Sulfolobus* sp. strain 7 utilize ferredoxins as electron acceptors for pyruvate:ferredoxin oxidoreductase and 2-oxoglutarate:ferredoxin oxidoreductase, respectively (28–30). Recently, Zhang *et al.* purified the 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 and proposed that the enzyme physiologically participates in the ferredoxin-dependent redox system (10). In the present study, we partially purified the 2-oxoglutarate:ferredoxin oxidoreductase from *P. islandicum*, and tried to

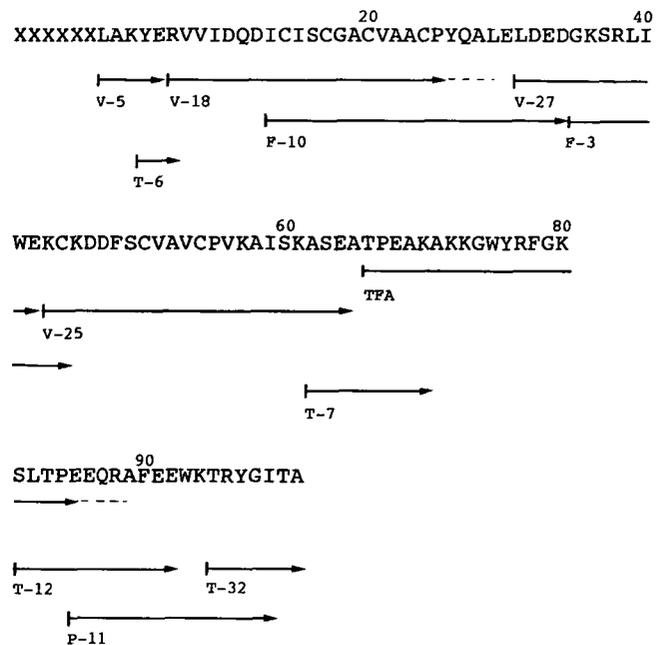


Fig. 5. Summary of the results of sequence studies on *P. islandicum* ferredoxin. V-5, V-18, V-25, and V-27, *Staphylococcus* V8-protease peptides; T-6, T-7, T-12 and T-32, tryptic peptides; F-3 and F-10, formic acid peptides; TFA, trifluoro acetic acid peptide; P-11, proline-specific endopeptidase peptide.

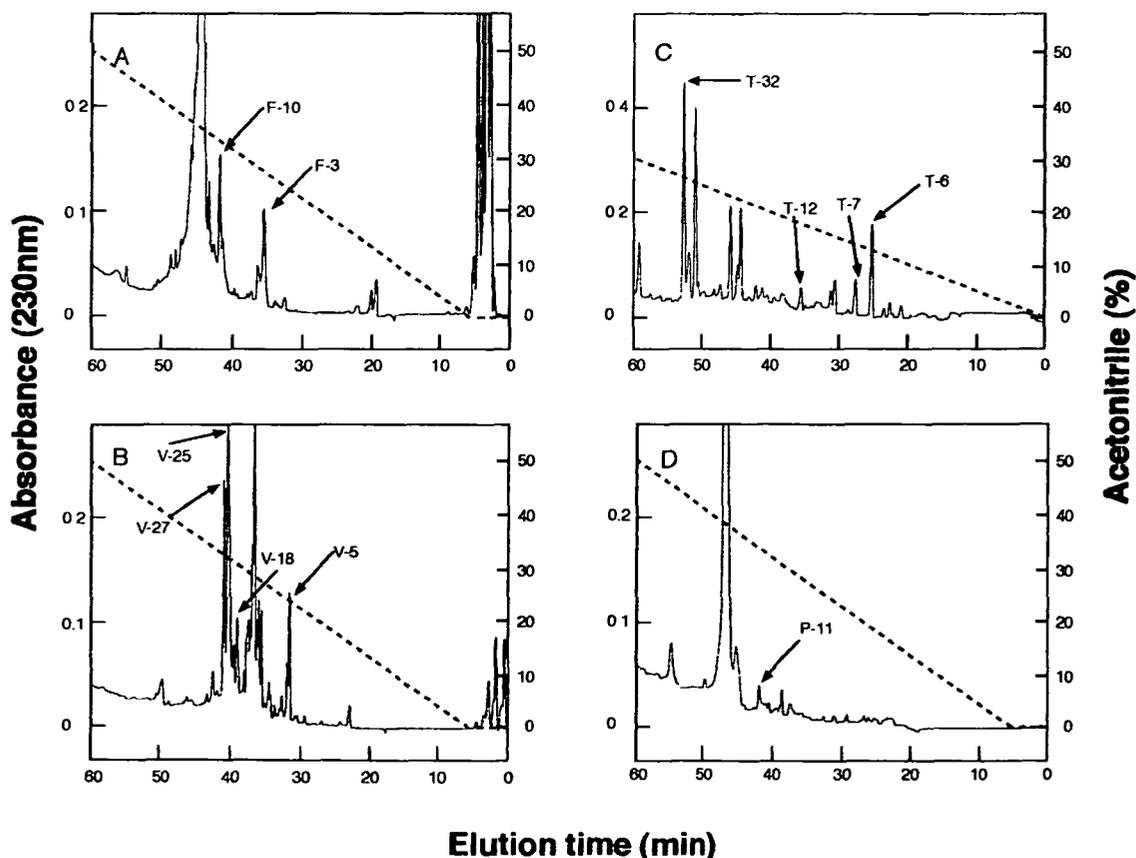


Fig. 4. Separation of digests of *P. islandicum* ferredoxin. The digests were subjected to HPLC under the conditions given under "MATERIALS AND METHODS." The dashed line shows the concentration of acetonitrile. (A) Formic acid digests; (B) *Staphylococcus* V8-protease digests; (C) trypsin digests; (D) proline-specific endopeptidase digests.

reconstitute the electron transfer between *P. islandicum* ferredoxin and 2-oxoglutarate at 70°C by the method described under "MATERIALS AND METHODS." Figure 3 shows the increase in absorbance at 550 nm of horse ferrocytochrome *c* in the presence of the *P. islandicum* ferredoxin and 2-oxoglutarate: ferredoxin oxidoreductase.

The cytochrome *c* was about 3-fold more rapidly reduced with the enzyme in the presence of ferredoxin than in its absence. The occurrence of the enzymatic activity in the absence of ferredoxin may be caused by the impure enzyme preparation.

As described above, the absorption spectra and EPR

A

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Pyrobaculum islandicum
Sulfolobus sp. strain 7
Thermoplasma acidophilum
Methanococcus thermolithotrophicus
Desulfovibrio vulgaris I
Methanosarcina thermophila
Clostridium acidurici
Clostridium pasteurianum
Azotobacter vinelandii I
Pyrococcus furiosus
Thermococcus litoralis
Thermotoga maritima
Clostridium thermoaceticum
Desulfovibrio africanus I
    
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XXXXXXXXLAK
GIDPQYRTN---RQVVGE[SG]EKVY-----GPLEPPKVLGI[
VKLEELDFKPKPIDEHFLENDKDYPTVG[NG]DVR[AE]GMQR[L]DADGKPYPTRLGI[
    
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P.is. YERVVIDODICIS--CGA[VAA]CPYQALELDEDGK-----SRLIWEKCKDDF--SCVAVCPVKAIKSKAS
S.sp.7 GTI[GV]DFD[CI]A--DGS[IN]A[FV]NVFQWYDTPGH-----PASEKKA[IP]VNEQACIFCM--ACUNVCEVAVEDVKP
T.ac. GTH[VAV]EWBC[CI]A--DCA[MD]VC[FV]NLYEWNLNPKSGTGNDRKIQKGEWNRK[RT]DKC[IP]VRESDCIFCM--ACESVCEVRAEKITP
Mc.th. S[VT]EYDKK[KG]P[EA]E[CN]A[CE]MEVF[EQ]G[Q]E[EV]V-----VAKEDDCTFCM--VCVDY[CE]TDA[ET]VKE
D.vu.I GWI[TV]DT[ER]CTG--DGE[VD]VC[VE]VY[EL]QDGKA-----VPVDEE[EL]GCE--SCV[EA]CEAG[ET]VVE
Ms.th. MVA[VNV]D[CT]G--CGS[VD]ECSAA[RI]S[EN]D[G]IA-----TVDESE[EL]GCG--SCEDACENNA[ET]TIE
C.ac. AYVINEACTS--CGA[CE]PECFVNA[IS]SGD[ER]VVI-----DADT[CI]E[CG]--ACAGVCEVD[AP]VQE
C.pa. AYKIA[DS]QVS--CGA[ASE]CFVNA[IS]QGDSIFVI-----DADT[CI]E[CG]--NCANVCEV[GA]P[V]QE
A.vi.I AFVVT[DN]G[IK]KYTD[SE]VCEVDCFYEGPNFLVI-----HPDE[CI]DCA--LCEPE[CA]Q[AF]S[ED]
P.fu. AWK[VS]VD[DT]CIG--DAI[AS]L[CE]DVFEMNDEGKAQP-----KVEVIEDE[EL]YNCA--KEAME[CE]V[SAT]TIEE
T.li. MKV[SV]DKD[AC]IG--CEVCASICE[DV]FEMDDG--KAK-----AEVA[ET]DLECA--KEA[AE]S[CE]T[CA]ETVE
T.ma. MKV[RV]DAD[AC]IG--CGV[EN]L[CE]DVFQLGEDG--KAK-----VE[Q]P[ET]DLPCA--KDA[AD]S[CE]T[CA]SVEE
C.th. MKV[TV]D[DT]CIA--CGT[IDL]CE[SV]FDWDEGLSHV-----IVDEVPEGAEDSCA--RES[ME]N[CE]P[ET]E[ET]KEV
D.af.I ARK[FY]VD[DT]CIA--CES[VE]IA[EG]AFAMDPEIE[EA]Y-----VKDVE[GA]SQEEVEEAMDTCEVQS[EE]
    
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P.is. ATPEAKAKKGWYRFGKSLTPEEQRAFEW[K]TRYGITA
A.vi.I VPEDMQEFIQLNAELAEVWP[N]ITEKKDPLPDAEDWDG[V]GKGLQHLER
    
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B

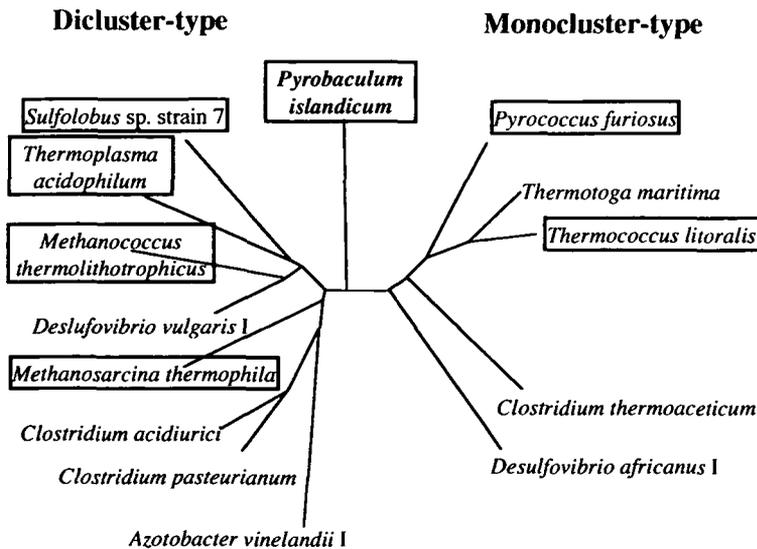


Fig. 6. (A) Amino acid sequence alignment of ferredoxins. All amino acid sequence data shown in the figure are from the GenEMBL, PIR, and SWISS-PROT databases, except those for the ferredoxin from *P. islandicum*. The boxed microorganisms are archaea. Residues identical with ones in the *P. islandicum* ferredoxin are shaded, and cysteine residues of the Fe-S cluster are denoted by white letters on a black background. The ligand residues to the zinc center in thermoacidophilic archaea are boxed. (B) A phylogenetic tree of ferredoxins. The branch lengths were calculated by means of the parsimony and neighbor-joining methods on the basis of the amino acid sequences of ferredoxins. The boxed microorganisms are archaea.

spectra indicated that the *P. islandicum* ferredoxin has a [3Fe-4S] cluster and a [4Fe-4S] cluster in the monomeric form. In the case of the [3Fe-4S] [4Fe-4S] type ferredoxin of *Sulfolobus* sp. strain 7, only the [3Fe-4S] cluster is reduced by 2-oxoacid:ferredoxin oxidoreductase, while the extremely low potential [4Fe-4S] cluster remains in the oxidized state (25). Indeed, the redox potential of the [4Fe-4S] cluster of the *Sulfolobus* sp. strain 7 ferredoxin (-530 mV) is lower than that of (CO₂ and succinylCoA)/(2-oxoglutarate and CoA) (-468 mV). Similar observations have been reported for *Clostridium pasteurianum*- and *Clostridium acidurici*-2[4Fe-4S] type dicluster ferredoxins, both of which are partially reduced with hydrogen and hydrogenase (31, 32). Thus, in some of the dicluster-type ferredoxins, only one Fe-S cluster is used as a single electron carrier *in vivo*. Therefore, it seems likely that one of the iron-sulfur clusters participates in the reaction with the *P. islandicum* 2-oxoglutarate:ferredoxin oxidoreductase.

Amino Acid Sequence of the *P. islandicum* Ferredoxin—After chemical digestion of the ferredoxin with formic acid as described under "MATERIALS AND METHODS," two peptides, F-3 and F-10, were obtained in pure states on fractionation on a Cosmosil 5C18 column (Fig. 4A). Peptide F-3 was sequenced up to residue 11. The *S. aureus* V8-protease-cleaved peptides (V-5, V-18, V-25, and V-27), TPKC-trypsin-cleaved peptides (T-6, T-7, T-12, and T-32), and proline-specific endopeptidase cleaved peptide (P-11) were obtained in pure states by fractionation on a Cosmosil 5C18 column (Fig. 4, B-D), and completely sequenced by the method as described under "MATERIALS AND METHODS."

Recently, Hulmes and Pan reported that polypeptides are selectively cleaved by TFA (33). In the present study, the ferredoxin was treated with TFA to deblock *N*-acetylserine or *N*-acetylthreonine as described under "MATERIALS AND METHODS." After TFA treatment, we had one TFA-polypeptide and thus could determine the amino acid sequence.

Although the N-terminal sequence of the *P. islandicum* ferredoxin was analyzed with a gas phase protein sequencer, no amino acids were detected, indicating that the N-terminus of the *P. islandicum* ferredoxin was blocked. Accordingly, we tried to deblock fMet, pyroglutamate, *N*-acetylserine, and *N*-acetylthreonine by conventional methods, as described under "MATERIALS AND METHODS." However, no amino acids were detected with the gas phase protein sequencer.

The results of sequence studies on the ferredoxin are summarized in Fig. 5. The total number of amino acid residues of the *P. islandicum* ferredoxin was 101, giving a molecular weight of 11,214. On the other hand, the molecular weight of the ferredoxin was estimated to be 11,910 on mass spectrometry. These results strongly indicate that the ferredoxin has more 6–8 amino acids at its N-terminus.

Sequence Analysis and Phylogenetic Tree of Archaeal Ferredoxins—Figure 6A shows the amino acid sequence of the *P. islandicum* ferredoxin aligned with those of ferredoxins from other organisms. Considering the C-terminal extension and the composition of the [Fe-S] clusters, the *P. islandicum* ferredoxin seems to be similar to *Azotobacter vinelandii* ferredoxin I, although the homology between the

two ferredoxins is low. *A. vinelandii* Fd I has two clusters, one comprising a [3Fe-4S] core co-ordinated by the first Fe-S binding motif, which consists of ⁸Cys, ¹⁶Cys, and ⁴⁹Cys, and the other having a [4Fe-4S] core co-ordinated by the second Fe-S binding motif, which consists of ²⁰Cys, ³⁹Cys, ⁴²Cys, and ⁴⁵Cys (34). However, it should be noted that ¹³Tyr and ⁴²Cys in *A. vinelandii* Fd I are replaced with cysteine and aspartate in the *P. islandicum* ferredoxin, respectively. Therefore, it seems likely that the [4Fe-4S] core is bound at the first motif and the [3Fe-4S] core is bound at the second motif. This is a matter for future investigation.

Figure 6B shows a phylogenetic tree of ferredoxins determined by means of the parsimony and neighbor-joining methods. The ferredoxins can be divided into two distinct subdivisions, dicluster and mono-cluster types. In the present study, we determined the amino acid sequence of the *P. islandicum* ferredoxin and investigated the phylogenetic relationship between bacterial ferredoxins. On the basis of the results of biochemical studies, the *P. islandicum* ferredoxin can be classified as a di-cluster type ferredoxin. However, the ferredoxin cannot be included in either the di-cluster type or the mono-cluster type, as shown in Fig. 6B. Therefore, the heat-stable ferredoxin from *P. islandicum* may be a novel iron-sulfur protein that has not previously been found in Archaea or Bacteria.

We would like to thank Dr. T. Iwasaki (Nippon Medical School) for the valuable discussions. We also thank Dr. Y. Isogai, Prof. Dr. T. Iizuka (Institute of Physical and Chemical Research), and Prof. Dr. T. Sakurai (Institute of Molecular Science) for their kind help in the EPR analysis, N. Wakiya (Tokyo Institute of Technology) for the metal content analyses by means of inductively coupled plasma atomic emission spectrometry, and Dr. E. Okumura (Tokyo Institute of Technology) for the mass spectrometry.

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