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Purifications and Characterizations of a Ferredoxin and Its Related 2-Oxoacid:Ferredoxin Oxidoreductase from the Hyperthermophilic Archaeon, *Sulfolobus solfataricus* P1

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The coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductase and ferredoxin (an effective electron acceptor) were purified from the hyperthermophilic archaeon, *Sulfolobus solfataricus* P1 (DSM1616). The purified ferredoxin is a monomeric protein with an apparent molecular mass of approximately 11 kDa by SDS-PAGE and of 11,180 ± 50 Da by MALDI-TOF mass spectrometry. Ferredoxin was identified to be a dicluster, [3Fe-4S][4Fe-4S], type ferredoxin by spectrophotometric and EPR studies, and appeared to be zinc-containing based on the shared homology of its N-terminal sequence with those of known zinc-containing ferredoxins. On the other hand, the purified 2-oxoacid: ferredoxin oxidoreductase was found to be a heterodimeric enzyme consisting of 69 kDa α and 34 kDa β subunits by SDS-PAGE and MALDI-TOF mass spectrometry. The purified enzyme showed a specific activity of 52.6 units/mg for the reduction of cytochrome c with 2-oxoglutarate as substrate at 55°C, pH 7.0. Maximum activity was observed at 70°C and the optimum pH for enzymatic activity was 7.0 -8.0. The enzyme displays broad substrate specificity toward 2-oxoacids, such as pyruvate, 2-oxobutyrate, and 2-oxoglutarate. Among the 2-oxoacids tested (pyruvate, 2-oxobutyrate, and 2-oxoglutarate), 2-oxoglutarate was found to be the best substrate with K_m and k_{cat} values of 163 μM and 452 min^{-1}, respectively. These results provide useful information for structural studies on these two proteins and for studies on the mechanism of electron transfer between the two.

Keywords: Archaea, Ferredoxin, 2-Oxoacids:ferredoxin oxidoreductase, *Sulfolobus solfataricus*, Thermostability

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Introduction

Ferredoxins are present in all organisms, from bacteria and archaea to higher eukaryotes. They are small and acidic iron-sulfur proteins with prosthetic groups, composed of iron and sulfur atoms, which are referred to as iron-sulfur clusters. The ferredoxins function as intracellular electron carriers with a low reduction potential (Mortenson *et al*., 1962; Beinert, 1990; Johnson, 1994). In addition to their roles in electron transfer reactions, iron-sulfur clusters are also now known to participate in the activation of substrates (Beinert *et al*., 1996; Broderick, *et al*., 1997), the stabilization of radicals and structures (Howard and Rees, 1996), the protection of proteins from enzymes (Grandoni *et al*., 1989), and the storage of iron and sulfur (Thauer and Schonheit, 1982), in addition, they act as sensors of iron, dioxygen, the superoxide ion (O_2^-), and possibly nitric oxide (Hentze and Kunh, 1996), and participate in gene expression (Michaels *et al*., 1990).

Ferredoxins are classified into three groups according to the types and numbers of their Fe-S clusters. The first group consists of monomeric (ca. 90-130 residues) proteins containing one [2Fe-2S] cluster, which includes ferredoxins that transfer electrons in redox chains linked to hydroxylases and oxygenases in a wide range of organisms (from bacteria to mammals) (Grinberg *et al*., 2000), and which includes plant and algal ferredoxins that function as electron carriers in photosynthesis (Matsubara and Sacki, 1992). The other two groups consist of ubiquitous proteins containing [3Fe-4S]/[4Fe-4S] and are typically found in bacteria. They may differ in cluster type (3Fe or 4Fe), number (one or two), and length of polypeptide chain (up to over 100 residues), and include the relatively small (ca. 55 residues) 2[4Fe-4S] ferredoxins that were first isolated from anaerobic bacteria (Mortenson *et al*., 1962).

Ferredoxins are widely distributed in archaea, and occur in methanogens, halophiles, and thermophiles (Kerscher and Oesterhelt, 1981; Daas *et al*., 1994; Breton *et al*., 1995). To

date the major types of clusters found in archaeal ferredoxins are as follows: [2Fe-2S] cluster found in a ferredoxin of *Sulfolobus solfataricus* (Kounosu *et al*., 2004), [4Fe-4S] cluster in a ferredoxin of *Pyrococcus furiosus* (Aono *et al*., 1989), and [3Fe-4S] [4Fe-4S] di-clusters in a ferredoxin of *Acidianus ambivalens* (Gomes *et al*., 1998), *Desulfurolobus ambivalens* (Teixeira *et al*., 1995), *Sulfolobus acidocaldarius* (Breton *et al*., 1995), *Sulfolobus* sp. strain 7 (Iwasaki *et al*., 1994), and *Thermoplasama acidophilum* (Iwasaki *et al*., 1997).

Although most thermophilic archaea are anaerobic organisms due to the scarcity of oxygen in their environments, one of the characteristic features in the central metabolic pathways of aerobic and anaerobic archaea is that ferredoxin functions as a physiological electron acceptor of the archaeal coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductase (Kerscher *et al*., 1982). Coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductases (100-300 kDa) in archaea (Kerscher and Oesterhelt, 1981; Blamey and Adams, 1993; Kunow *et al*., 1995; Zhang *et al*., 1996) play central roles in the decarboxylation of pyruvate and 2-oxoglutarate, rather than the NAD^+-dependent 2-oxoacid dehydrogenase multienzyme complexes (2,000-7,000 kDa) which play similar roles in bacteria and eukaryotic mitochondria (Koike and Koike, 1976), and they contain one TPP and at least one [4Fe-4S] cluster (per protomer) as prosthetic groups (Zhang *et al*., 1996). The cofactor composition of archaeal enzymes is similar to that of enzymes from fermentative and phototrophic obligatory anaerobic bacteria (Meinecke *et al*., 1989), several photosynthetic bacteria (Brostedt and Nordlund, 1991), and anaerobic and amitochondrial protozoa (Williams *et al*., 1987). The 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 is known to consist of 70-kDa α and 37-kDa β subunits, and to contain one TPP and one [4Fe-4S] cluster, and two Mg atoms per $\alpha\beta$ structure (Zhang *et al*., 1996).

S. solfataricus P1 (DSM1616) belongs to the thermoacidophilic archaea, which inhabit sulfur-rich volcanic hot springs, a high temperature/low pH environment (Brock *et al*., 1972; Rosa *et al*., 1975; Zillig *et al*., 1980). In this paper, we report for the first time on the purification and characterization of thermostable ferredoxin and 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1 strain. The purified ferredoxin was identified as an electron carrier containing dicluster, [3Fe-4S] [4Fe-4s], and the purified 2-oxoacid:ferredoxin oxidoreductase was found to be a heterodimeric enzyme, which showed a fastest turnover rate of 2-oxoglutarate than of the two other oxoacids, pyruvate and 2-oxobutyrate.

Materials and Methods

Materials. *Sulfolobus solfataricus* P1 (DSM1616) was purchased from the American Type Culture Collection. Yeast extract was purchased from Difco Laboratories and DEAE-Sephacel, Phenyl Sepharose CL-4B, Q-Sepharose Fast Flow, Superdex 200, Sephadex G-50, and Blue Sepharose 6 fast flow were from Pharmacia Biotech. 2-Oxoglutarate and pyruvate were purchased from Biochemical. All other chemicals used were purchased from Sigma (St. Louis) unless otherwise stated.

Organism and growth conditions. *S. solfataricus* P1 (DSM1616) was aerobically cultivated at 75°C/pH 4.0 in a 5 L fermentor with moderate stirring (120 rpm). The medium containing 0.22% (w/v) yeast extract and the mineral bases was as described for ATCC media formulation (Zillig *et al*., 1980). Cells were harvested by centrifugation (4,000 × *g*) for 45 min at 4°C, and stored at −20°C.

Purification of ferredoxin and 2-oxoacid:ferredoxin oxidoreductase. All purification steps were performed at 4°C. A 15 g batch of frozen cells was thawed and resuspended in 10 mM sodium phosphate buffer (pH 7.0) (buffer A) to produce a 20% (wt/vol) homogeneous solution. Cells were disrupted by passing them three times through a chilled FRENCH Pressure Cell (Thermo Spectronic Inc.) at a pressure of 1,200 psi. Cell debris was precipitated by centrifugation (150,000 × *g*) for 90 min at 4°C. Precipitates were composed of two layers. The soft upper layer was carefully collected and resuspended in buffer A, and then recentrifuged using the same conditions. Supernatant fractions after centrifugation were combined and dialyzed three times against 5 liters of buffer A for 24 h to remove salts and to enhance the recovery of iron-sulfur proteins during the DEAE-Sephacel column chromatography purification step. The dialyzed solutions are referred to as cell extracts.

Ferredoxin was purified by comparing the absorbances of collected column chromatography fractions at 283 nm and 405 nm. Briefly, cell extract was applied to a column of DEAE-Sephacel (3.0 × 20 cm) equilibrated with buffer A. The column was then washed with 500 ml of buffer A until absorbance at 280 nm reached the base line, and was then eluted with 400 ml of a linear gradient of 0 to 0.15 M NaCl in buffer A. If the brown colored sample remained in the column, further elution was carried out with 300 ml of 0.15 M NaCl in buffer A. Fractions containing the dark brown ferredoxin were pooled, adjusted to 1 M using powdered ammonium sulfate, and then loaded onto a Phenyl Sepharose column (1.5 × 15 cm) equilibrated with buffer A containing 1 M ammonium sulfate. The column was then washed using 200 ml of the same equilibration buffer and eluted with 300 ml of a reverse linear gradient of 1 to 0 M ammonium sulfate in buffer A. The fractions containing ferredoxin were collected and dialyzed three times against 5 liters of buffer A for 24 h to remove the ammonium sulfate. The dialyzed protein solution was then applied to a Q-Sepharose column (1.5 × 15 cm) equilibrated with buffer A, washed with 200 ml of buffer A, and eluted with a 300 ml linear gradient of 0 to 0.3 M NaCl in buffer A. The collected protein solution, containing ferredoxin, was concentrated by using an Amicon concentrator (Amicon Centriprep YM-10, Millipore), and the concentrated sample was applied to a Sephadex G-50 column (1.5 × 50 cm) equilibrated with buffer A containing 20 mM NaCl and eluted with the same equilibration buffer. Fractions containing ferredoxin were collected and dialyzed twice against 5 L of buffer A to remove salt. Dialyzed protein solutions are referred to as purified ferredoxin solutions, and if required, were concentrated using an Amicon concentrator (Amicon Centriprep YM-10, Millipore).

To purify 2-oxoacid:ferredoxin oxidoreductase, cell extract was applied to a DEAE-Sephacel column (3.0 × 20 cm) equilibrated with buffer A. The column was then washed with 300 ml of buffer

A until the absorbance at 280 nm reached base line, and then eluted with 400 ml of a linear gradient of 0 to 0.15 M NaCl in buffer A. Fractions containing 2-oxoacid:ferredoxin oxidoreductase activity were pooled, powdered ammonium sulfate was added to the pooled protein solution to 0.5 M, which was then loaded onto a Phenyl Sepharose column (1.5 × 15 cm) equilibrated with buffer A containing 0.5 M ammonium sulfate. The column was then washed with 200 ml of the same equilibration buffer and eluted with 300 ml of a reverse linear gradient of 0.5 to 0 M ammonium sulfate in buffer A. Fractions containing 2-oxoacid:ferredoxin oxidoreductase activity were collected, and concentrated using an Amicon concentrator (Amicon Centriprep YM-10, Millipore). The concentrated protein solution was then applied to a Superdex 200 column (1.5 × 60 cm) equilibrated with buffer A containing 0.2 M NaCl and eluted with the same equilibration buffer. Fractions containing 2-oxoacid:ferredoxin oxidoreductase activity were collected and dialyzed three times against 5 liters of buffer A for 24 h to remove salt. The dialyzed protein solution was then applied to a Q-Sepharose column (1.5 × 15 cm) equilibrated with buffer A, washed with 200 ml of buffer A, and then eluted with 300 ml of a linear gradient of 0 to 0.3 M NaCl in buffer A. Fractions containing 2-oxoacid:ferredoxin oxidoreductase activity were collected and dialyzed as described above. The dialyzed sample was then loaded onto a Blue Sepharose column (1.0 × 10 cm) equilibrated with buffer A and eluted with 300 ml of a linear gradient of 0 to 0.3 M NaCl in buffer A. Fractions containing 2-oxoacid:ferredoxin oxidoreductase activity were collected and then dialyzed as described above. Dialyzed protein solutions are referred to as purified 2-oxoacid:ferredoxin oxidoreductase solutions.

Measurement of enzymatic activity. Standard 2-oxoacid:ferredoxin oxidoreductase activity assays were carried out at 55°C by monitoring changes in absorbance at 550 nm, due to the ferredoxin-dependent reduction of a horse heart cytochrome c in the presence of 2-oxoglutarate as a substrate, by using a modified version of the method described by Kerscher *et al.* (Kerscher *et al.*, 1982; Iwasaki and Oshima, 2001). Briefly, the enzyme reaction was started by adding 0.1 ml of the purified enzyme solution (50 μg/ml) to 0.1 ml of prewarmed purified ferredoxin solution (260 μg/ml) and 0.8 ml of prewarmed 100 mM sodium phosphate buffer (pH 7.0) containing 20 μM horse heart cytochrome c, 20 μM coenzyme A, and 4 mM 2-oxoglutarate. For all measurement, the effect of cytochrome c nonenzymatic reduction was taken into consideration and subtracted from values determined in the presence of enzyme. Measurements were carried out at least three times. Standard deviations never exceeded 10% of the mean values. One enzyme unit was defined as the amount of enzyme required to reduce 1 μmol of cytochrome c per min at 55°C. A reduction of cytochrome c by 1 nmol corresponded to an A_{550nm} increase of 0.021 (Kerscher *et al.*, 1982). To determine substrate specificities and the effects of ferredoxin on the activities of purified enzyme toward other kinds of 2-oxoacids, namely, glyoxylate, pyruvate, and 2-oxobutyrate, in addition to 2-oxoglutarate were also examined in the absence and presence of ferredoxin (Zhang *et al.*, 1996).

Effects of pH and temperature. The optimum pH and temperature for purified 2-oxoacid:ferredoxin oxidoreductase activity were determined using a standard 2-oxoacid:ferredoxin oxidoreductase assay using 2-oxoglutarate as a substrate. The effect of pH on enzyme activity was examined at 55°C in the pH range, 4.0 to 11.0. The following buffers (100 mM) were used: sodium acetate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), Tris/HCl (pH 8.0 to 9.0), and sodium bicarbonate (pH 9.0 to 11.0). The effect of temperature on enzyme activity was investigated at the optimum pH (pH 7.0) at temperatures ranging from 10°C to 70°C.

Analytical methods. Absorption spectra were recorded using a Shimadzu UV-2401PC spectrophotometer equipped with a cell holder having a circulating water jacket system. EPR measurements were carried out using a JEOL JES-TE200 spectrometer equipped with a Heli-Tran cryostat system (Model LT-30M, Air Products), in which temperature was monitored using a Scientific Instruments series 5500 temperature indicator/controller (Aasa and Vänngärd, 1975).

Native PAGE (Laemmli, 1970) was performed using a 10% or 12.5% (w/v) gels to assess enzyme purity. Molecular weights of the purified ferredoxin and 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1 were determined using a low molecular weight standard (Amersham Bioscience) on 12.5% and 10% SDS-PAGE, respectively (Laemmli, 1970). Gels were stained with silver (Ohsawa and Ebata, 1983). The molecular weights of the native proteins were also determined by HPLC [GPC column (Protein-Pak™ 300SW, 7.8 × 300 mm, Waters)] using molecular weight standards (Sigma MW-GF-200 kit) and by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Voyager DE STR, Applied Biosystems) using cytochrome C (12 kDa) and BSA (66 kDa) for calibration.

N-terminal amino acid sequences were analyzed using an automatic amino acid sequencer (Hewlett Packard protein sequencer HP241) according to the manufacturer's instructions.

Protein concentrations were determined using the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Results and Discussion

Purification and physical properties of ferredoxin from *S. solfataricus* P1. To establish an assay for 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1, we purified thermostable ferredoxin from the same strain. The purification procedure described here yielded electrophoretically homogeneous ferredoxin in four steps starting from cell extracts of *S. solfataricus* P1 (DSM1616), as described in "Materials and Methods". Approximately 2.5-4 mg of the purified ferredoxin was routinely obtained from 15 g batch of frozen cells. The homogeneity of the purified ferredoxin was confirmed on SDS-PAGE gel as a single band of approximately 11 kDa (Fig. 1A). A purity index (A_{408}/A_{280}) of the purified ferredoxin was 0.71 (Fujii et al., 1991), and its molecular weight was determined using a sizing HPLC column to be approximately 11 kDa, indicating that the enzyme was monomeric and showing good agreement with the MALDI-TOF-MS determined molecular mass (Fig. 1B).

Fig. 2 shows optical absorption spectra of the purified ferredoxin (0.17 mg/ml in 100 mM sodium phosphate buffer,

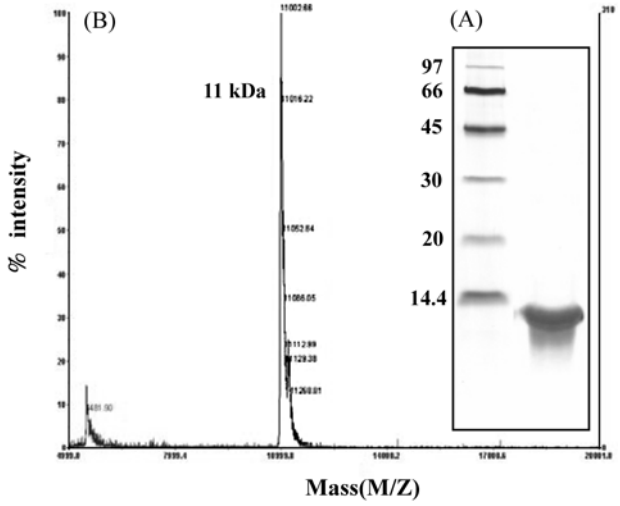

Fig. 1. Electrophoretic and mass spectrometric analyses of the purified ferredoxin from *S. solfataricus* P1. (A) A purified ferredoxin with molecular size markers on a 12.5% SDS-PAGE gel. (B) MALDI-TOF-MS spectrum of the ferredoxin.

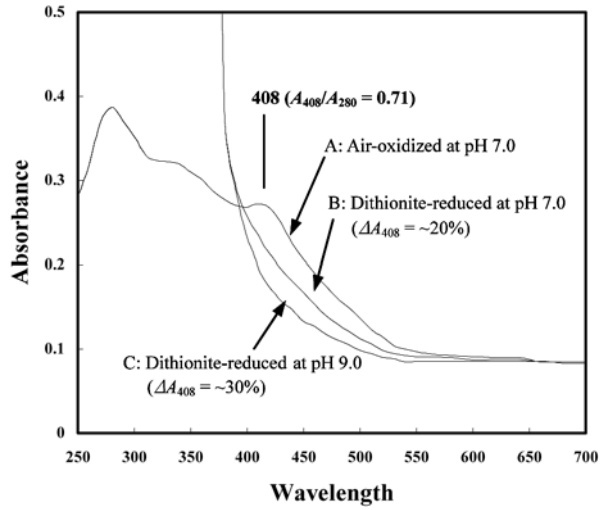

Fig. 2. Ultraviolet-visible absorption spectra of the purified ferredoxin from *S. solfataricus* P1. Ferredoxin (0.17 mg/ml) was dissolved in 100 mM sodium phosphate buffer, pH 7.0 (A and B), or in 100 mM Tris-Cl buffer, pH 9.0 (C): A, air-oxidized form as prepared; B and C, reduced form prepared by adding excess sodium dithionite.

pH 7.0), which are typical for iron-sulfur containing proteins. Purified ferredoxin displayed an absorption peak at 280 nm and a broad peak at around 408 nm in the air-oxidized state (Fig. 2A). The spectrum of dithionite-reduced ferredoxin was obtained by re-scanning after adding an excess of solid sodium dithionite to an air-oxidized sample solution. As shown in Fig. 2B, a ca. 20% decrease in absorbance was observed at 408 nm. The addition of more sodium dithionite did not cause a further reduction in absorbance at 408 nm. However, the addition of sodium dithionite to an air oxidized

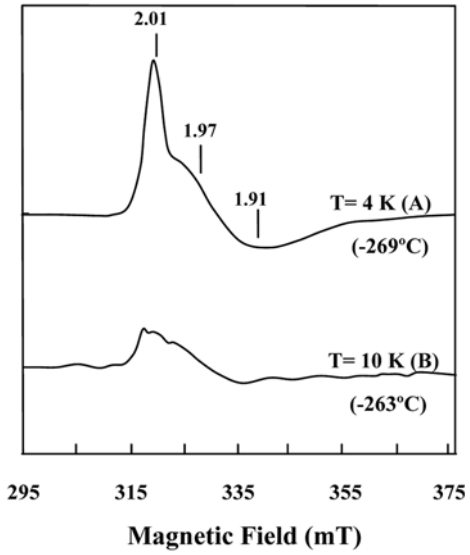

Fig. 3. Temperature dependence by EPR of the air-oxidized form of the purified ferredoxin from *S. solfataricus* P1. The sample was dissolved in 10 mM sodium phosphate buffer (pH 7.0). The instrument settings for EPR spectroscopy were; temperature, 4 K (A) or 10 K (B); microwave power 1.02 mW; and modulation amplitude 0.79 mT; *g* values are indicated in the figure.

ferredoxin in 100 mM Tris-Cl buffer, pH 9.0, induced a further reduction at 408 nm band of approximately 30% of its original absorbance (Fig. 2C). These observations indicate that the purified ferredoxin was in a partially reduced state under experimental conditions, since most bacterial-type ferredoxins show a reduction of 40% or more at 400 nm in the fully reduced state (Blamey *et al*., 2000; Green *et al*., 2003). In addition, it appears likely that *S. solfataricus* ferredoxin has an iron-sulfur cluster of unusually low redox potential with pH-dependence. From these results it seems that ferredoxin from *S. solfataricus* P1 is similar to dicluster-type ferredoxins that possess one low potential iron-sulfur cluster, such as, those from archaea, *Sulfolobus* sp. strain 7 (Iwasaki *et al*., 1994), *Pyrobaculum islandicum* (Nakajima *et al*., 1998), *D. ambivalens* (Teixeira *et al*., 1995), and *S. acidocaldarius* (Breton *et al*., 1995). The purified ferredoxin showed extreme thermostability, as judged by an unchanged optical absorption spectra after incubation for 2 h at 75°C or for over 48h at 55 °C at pH 7.0. In addition, it is stable at 55°C across a broad pH range, i.e., from 5 to 11.

Fig. 3 displays the effect of temperature on the X-band EPR spectra of the air-oxidized form of the purified ferredoxin. The EPR spectrum at 4K exhibited a sharp peak at $g = 2.01$, a broad trough around $g = 1.91$ with an extended tail toward higher magnetic field, and a distinct shoulder at $g = 1.97$. This single sharp peak, with a g_{max} of 2.01, corresponds to a center with an extremely rapid spin relaxation rate. However, this spectrum could not be detected clearly at 10K. Moreover, the spectral shape was strongly temperature dependent, with noticeable line broadening even at temperatures only slightly

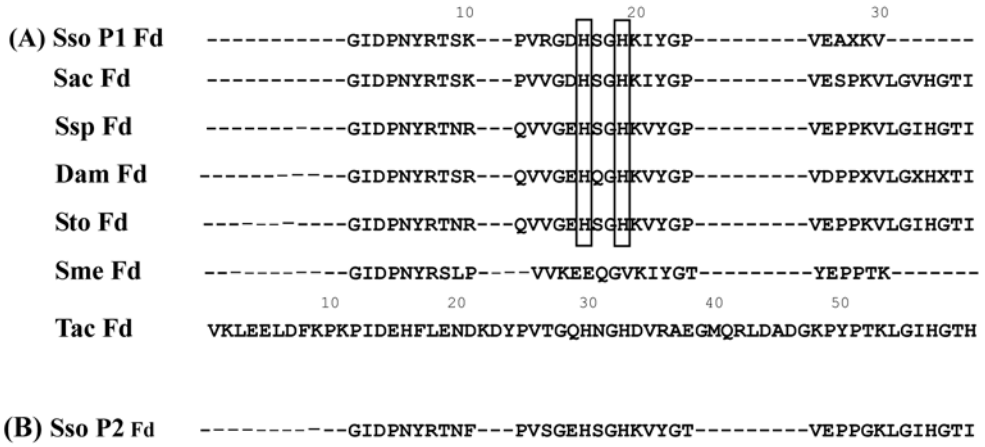

Fig. 4. Multiple alignments of the N-terminal amino acid sequence of the purified ferredoxin from *S. solfataricus* P1 (DSM 1616) (Sso P1 Fd) with those of ferredoxins from various other archaea such as Sac (*S. acidocaldarius*), Ssp (*Sulfolobus* sp. strain 7), Dam (*D. ambivalence*), Sto (*S. Tokodaii*), Sme (*S. metallicus*), and Tac (*T. acidophilum* DSM1738) (A), and with that of a putative ferredoxin deduced from genes found in the complete genome of *S. solfataricus* P2 (Sso P2) (B). The boxes indicated possible ligand residues related to the zinc binding motif.

above 4K. These results strongly suggest that at least one [3Fe-4S] cluster is present in the purified ferredoxin. Similar signals were observed for the [3Fe-4S] cluster of dicluster (7Fe) ferredoxins from *Sulfolobus* sp. strain 7 (Iwasaki *et al*., 1994), *S. acidocaldarius* (Breton *et al*., 1995), *Thermus thermophilus* (Hagen *et al*., 1985), and *D. ambivalens* (Teixeira *et al*., 1995).

The sequence of 30 amino acid residues at the N-terminus of the purified ferredoxin from *S. solfataricus* P1 was determined and compared with the sequence data retrieved from the Entrez server at NCBI (http//www.ncbi.nlm.nih.gov/Entrez/) and from papers on ferredoxins from other archaea (Fig. 4). The N-terminal amino acid sequence of ferredoxin from *S. solfataricus* P1 (DSM 1616) showed highest similarity (93% identity) with that of *S. acidocaldarius* ferredoxin containing diclusters, [3Fe-4S] [4Fe-4S] (Minami *et al*., 1985; Breton *et al*., 1995). In addition, it displayed more than 50% similarity with ferredoxins containing dicluster, [3Fe-4S] [4Fe-4S], from *Sulfolobus* sp. strain 7, *D. ambivalens, Sulfolobus Tokodaii,* and *Sulfolobus metallicus* belonging to the *Sulfolobaceae* family (Wakagi *et al*., 1996; Iwasaki *et al*., 1997; Gomes *et al*., 1998; Iwasaki and Oshima, 2001). However, it showed little similarity with dicluster containing ferredoxins, [3Fe-4S] [4Fe-4S], from *T. acidophilum* or single cluster containing ferredoxins, [4Fe-4S], from *P. furiosus*, which belong to other thermophilic archaea (Wakagi *et al*., 1996; Gomes *et al*., 1998). The N-terminal sequence of ferredoxin from *S. solfataricus* P1 was not identical to that of a putative ferredoxin composed of 103 amino acids derived from the genomic sequence of *S. solfataricus* P2 (She *et al*., 2001); these shared a similarity of 69%. In addition, the purified ferredoxin appears to be a zinc-containing protein, because it contains signature histidine residues in its N-terminal sequence, like the amino acid sequences of other zinc-containing ferredoxins (Iwasaki *et al*., 1997).

Purification and physical properties of 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1. As shown in Table 1, 2-oxoacid:ferredoxin oxidoreductase was purified to electrophoretic homogeneity using five steps starting from *S. solfataricus* P1 (DSM1616) cell extract, as described in "Materials and Methods". The 2-oxoacid:ferredoxin oxidoreductase was purified 44-fold with a yield of 6%, and showed a specific activity of 52.6 units/mg for the reduction of cytochrome c at 55°C (pH 7.0) by standard enzyme assay using 2-oxoglutarate

Table 1. Summary of the purity of the 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1

Step	Protein (mg)	Activity (U)*	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude	137.7	167.1	1.2	100	1
DEAE-Sepharose	27	149.9	5.5	89	5
Phenyl- Sepharose	4	110.5	30.2	66	25
Superdex-200	2	63.1	35.7	38	29
Q- Sepharose	1	37.9	37.9	23	31
Blue- Sepharose	0.2	10.5	52.6	6	44

*One unit of the enzyme is defined as the amount of enzyme required to reduce 1 μmol of cytochrome c per min at 55°C. A cytochrome c reduction of 1nmol corresponded to an A_{550nm} increase of 0.021.

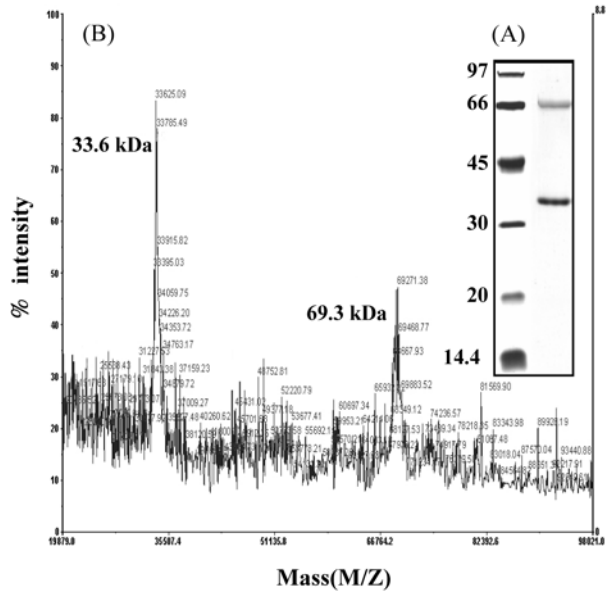

Fig. 5. Electrophoretic and mass spectrometric analyses of the purified 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1. (A) A purified 2-oxoacid:ferredoxin oxidoreductase with molecular size markers on a 10% SDS-PAGE gel. (B) MALDI-TOF-MS spectrum of 2-oxoacid:ferredoxin oxidoreductase.

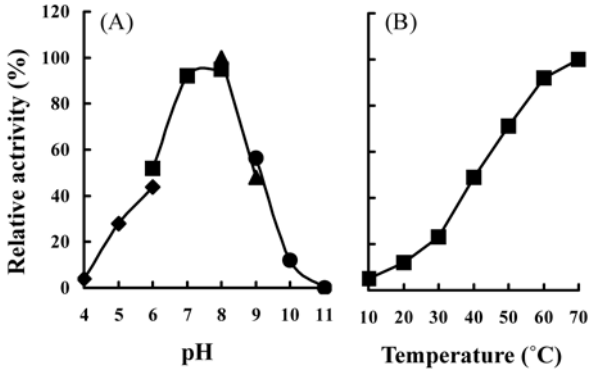

Fig. 6. Effect of pH and temperature on the activity of the purified 2-oxoacid:ferredoxin oxidoreductase. The effects of pH and temperature on the activity of the purified 2-oxoacid:ferredoxin oxidoreductase were determined spectrophotometrically using a standard 2-oxoacid:ferredoxin oxidoreductase assay using 2-oxoglutarate as a substrate, as described in "Materials and Methods". The enzyme activity was examined at 55°C and different pH's (A) or at pH 8.0 and different temperatures (B). Required pH's were obtained using 100 mM sodium citrate buffer (◆) in the pH range 5.0 to 6.0; 100 mM sodium phosphate buffer (■) in the pH range 6.0 to 8.0; 100 mM Tris-Cl buffer (▲) in the pH range 8.0 to 9.0, and 100 mM sodium bicarbonate buffer (●) in the pH range 9.0 to 11.0.

and the purified ferredoxin from *S. solfataricus* P1 (DSM1616) as substrates. The homogeneity of the purified enzyme was confirmed on native PAGE gel as a single band and by the presence of a single peak at approximately 103 kDa by HPLC using a sizing column (data not shown). The molecular size of the enzyme is similar to those of other 2-oxoacid:ferredoxin oxidoreductases from hyperthermophilic archaea such as *Sulfolobus* sp. strain 7 (103 kDa), *P. furiosus* (115 kDa), and *Archaeoglobus fulgidus* (120 kDa) and corresponds roughly to half the size of that of *Halobacterium halobium* enzyme (Kerscher and Oesterhelt, 1981; Zhang *et al*., 1996). The purified enzyme was analyzed by SDS-PAGE gel as a heterodimeric enzyme, with apparent molecular masses of 34 kDa and 69 kDa, respectively (Fig. 5A). Moreover, the molecular masses of these two subunits are in good agreement with those obtained by MALDI-TOF-MS analysis (Fig. 5B). The 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 (103 kDa) was previously reported to be a heterodimeric enzyme composed of 37 and 70 kDa (Zhang *et al*., 1996). In contrast, 2-oxoacid:ferredoxin oxidoreductases from other hyperthermophilic archaea such as *P. furiosus* (Zhang *et al*., 1996; Kletzin and Adams, 1996), *A. fulgidus* (Kunow *et al*., 1995), and *H. halobium* (Kerscher and Oesterhelt, 1981; Zhang *et al*., 1996) have been reported as heterotetrameric enzymes.

The effect of pH and temperature on 2-oxoacid:ferredoxin oxidoreductase activity was determined using cytochrome c at several different pHs and temperatures. The purified enzyme displayed maximum activity at pH 7.0-8.0 (Fig. 6A) and at 70°C (Fig. 6B). Activity assays were routinely performed for convenience using phosphate buffer, pH 7.0, and at 55°C, to characterize the purified enzyme, since horse heart cytochrome c is stable to 55°C.

The dependence of the purified enzyme on ferredoxin obtained from *S. solfataricus* P1 and its substrate specificity toward various 2-oxoacids, such as pyruvate, 2-oxobutyrate, and 2-oxoglutarate were investigated; results are summarized in Tables 2 and 3. As shown in Table 2, the cytochrome c reducing activity of the enzyme was negligible toward any of the 2-oxoacids in the absence of ferredoxin. Cytochrome c reducing activities toward these 2-oxoacids in the presence of cognate ferredoxin followed the order of 2-oxoglutarate > pyruvate > 2-oxobutyrate. Moreover, its enzymatic activity for 2-oxoglutarate was about three times higher than that for pyruvate, and about five times higher than that for 2-oxobutyrate. In contrast, no activity was detected toward glyoxylate either in the presence or absence of ferredoxin. These results indicate that the purified enzyme depends on ferredoxin for 2-oxoacid-dependent cytochrome c reduction and that it shows broad substrate specificity toward various 2-oxoacids. Our results are in agreement with a report that the homologous enzyme from *Sulfolobus* sp. strain 7 also depends on ferredoxin for cytochrome c reduction, but contrast with a report that the specific activities of the enzyme in the presence of cognate ferredoxin follow the order; 2-oxoglutarate > 2-oxobutyrate > pyruvate (Zhang *et al*., 1996). In addition, pyruvate:ferredoxin oxidoreductase from *H. halobium* accepts 2-oxobutyrate, pyruvate, and 2-oxoglutarate; in decreasing

Table 2. Effect of ferredoxin on 2-oxoacid:ferredoxin oxidoreductase activity

O ‖ R-C-COO- Alkyl group	Substrate (4 mM)	Relative activity of cytochrome c reduction (%)	
		- ferredoxin	+ 25 μM ferredoxin
-H	Glyoxylate	~1	~1
$-CH_3$	Pyruvate	4.3	31.2
$-CH_2-CH_2-CH_3$	2-Oxobutyrate	4.1	20.4
$-CH_2-CH_2-COO^-$	2-Oxoglutarate	4.5	100.0

The ferredoxin dependence of the 2-oxoacid:ferredoxin oxidoreductase reaction was examined using a standard assay in either the absence or presence (25 μM) of ferredoxin as described in "Materials and Methods".

Table 3. Kinetic parameters of 2-oxoacid:ferredoxin oxidoreductase for various 2-oxoacids

	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1} \cdot min^{-1}$)
2-Oxoglutarate	1.63×10^{-4}	4.52×10^{2}	2.77×10^{6}
Pyruvate	2.75×10^{-4}	1.44×10^{2}	5.24×10^{5}
2-Oxobutyrate	5.16×10^{-4}	0.93×10^{2}	1.80×10^{5}

order of specific activity (Kerscher and Oesterhelt, 1981). The substrate specificities of the purified enzyme toward various 2-oxoacids are summarized in Table 3. The highest K_m value of 516 μM was observed for 2-oxobutyrate, whereas the lowest K_m value of 163 μM was observed for 2-oxoglutarate. However, k_{cat} was highest for 2-oxoglutarate and lowest for 2-oxobutyrate. The specificity constant (k_{cat}/K_m) values for 2-oxoglutarate, 2-oxobutyrate, and pyruvate were 2.77×10^6, 5.24×10^5, and 1.80×10^5 $M^{-1} \cdot min^{-1}$, respectively. Moreover, the turnover of 2-oxoglutarate was 5 times that of pyruvate and more than 10 times that of 2-oxobutyrate. Our data indicate that the best 2-oxoacid substrate (from among the three tested) for 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1 is 2-oxoglutarate.

In conclusion, the present paper reports for the first time on the purification and characterization of thermostable ferredoxin and 2-oxoacid:ferredoxin oxidoreductase from *S. solfataricus* P1 strain. *S. solfataricus* ferredoxin was found to contain a dicluster, i.e., a [3Fe-4S] cluster and a [4Fe-4S] cluster, by spectroscopic analyses, and to possibly contain one zinc center by comparing its N-terminal amino acid sequence with those of well known zinc-containing ferredoxins. In addition, it is suggested that this *S. solfataricus* 2-oxoacid:ferredoxin oxidoreductase is αβ-type heterodimeric enzyme and shows broad substrate specificity toward 2-oxoacids, but a fastest turnover of 2-oxoglutarate. This new information should be useful for those performing structural studies on these two proteins and the study on the mechanism of electron transfer between ferredoxin and 2-oxoacid:ferredoxin oxidoreductase.

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