THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 51, Issue of December 17, pp. 36715–36721, 1999
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Printed in U.S.A.

Identification and Characterization of a Novel Ferric Reductase from the Hyperthermophilic Archaeon *Archaeoglobus fulgidus**

(Received for publication, June 18, 1999, and in revised form, August 20, 1999)

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***Archaeoglobus fulgidus*, a hyperthermophilic sulfate-reducing Archaeon, contains high Fe^{3+}-EDTA reductase activity in its soluble protein fraction. The corresponding enzyme, which constitutes about 0.75% of the soluble protein, was purified 175-fold to homogeneity. Based on SDS-polyacrylamide gel electrophoresis, the ferric reductase consists of a single subunit with a M_r of 18,000. The M_r of the native enzyme was determined by size exclusion chromatography to be 40,000 suggesting that the native ferric reductase is a homodimer. The enzyme uses both NADH and NADPH as electron donors to reduce Fe^{3+}-EDTA. Other Fe^{3+} complexes and dichlorophenolindophenol serve as alternative electron acceptors, but uncomplexed Fe^{3+} is not utilized. The purified enzyme strictly requires FMN or FAD as a catalytic intermediate for Fe^{3+} reduction. Ferric reductase also reduces FMN and FAD, but not riboflavin, with NAD(P)H which classifies the enzyme as a NAD(P)H:flavin oxidoreductase. The enzyme exhibits a temperature optimum of 88 °C. When incubated at 85 °C, the enzyme activity half-life was 2 h. N-terminal sequence analysis of the purified ferric reductase resulted in the identification of the hypothetical gene, *AF0830*, of the *A. fulgidus* genomic sequence. The *A. fulgidus* ferric reductase shares amino acid sequence similarity with a family of NAD(P)H:FMN oxidoreductases but not with any ferric reductases suggesting that the *A. fulgidus* ferric reductase is a novel enzyme.**

Acquisition of iron for assimilation into cellular protein is a universal trait of life. Iron, in its many inorganic and organic forms, is the fourth most abundant element on earth (1). However, at physiological pH and under aerobic conditions, iron forms hydroxides and oxyhydroxides at exceedingly low solubilities of 10^{-17} to 10^{-18} M (1 μM being the threshold concentration to support life) (2, 3). For this reason, organisms acquire insoluble Fe^{3+} by complexing it with ferric-specific chelators, *i.e.* siderophores (2). Depending on the organism, the reduction of complexed Fe^{3+} is accomplished either before or after transport into the cell. Ferric reductase catalyzes the reduction of complexed Fe^{3+} to complexed Fe^{2+} using NAD(P)H as the electron donor (4). The resulting Fe^{2+} is subsequently released and incorporated into iron-containing proteins (5).

Assimilatory ferric reductase activities have been identified and characterized in animals, plants, yeast, and bacteria. In general, these enzymes are monomeric to homotetrameric with molecular weights ranging from 26,000 to 69,000 per monomer. The majority of the eukaryotic ferric reductases is membrane-bound and contains cytochrome *b* as well as covalently or non-covalently bound flavin (6–9). In contrast, prokaryotic ferric reductases lack cytochromes and bound flavin but require exogenous FMN for optimal activity (10).

Dissimilatory ferric reductases catalyze the reduction of Fe^{3+} as the terminal electron acceptor in energy metabolism (11). Iron-reducing organisms are found among the Gram-negative and Gram-positive genera of the Proteobacteria. They include the following: *Shewanella putrifacience,* strain GS-15, *Geobacter metallireducens*, *Geobacter sulfurreducens*, *Desulfuromonas acetoxidans*, *Bacillus* species, *Thermotoga maritima*, and other isolated bacteria (12–14). Two cytochrome *c*-type proteins with metal reductase activity were purified from *G. sulfurreducens* and *D. acetoxidans*. They also reduced complexed Fe^{3+} (15, 16). Both *c*-type cytochromes are located in the periplasm of the respective bacterium. Recently, Vargas *et al.* (17) reported iron-reducing activities in cell suspensions of certain hyperthermophilic Archaea including *Archaeoglobus fulgidus*. The authors (17) demonstrated that the Archaeon, *Pyrobaculum islandicum*, is capable of utilizing Fe^{3+}-citrate as electron acceptor for growth.

Our knowledge about dissimilatory and assimilatory iron reduction in Archaea is scarce. Since little is known about the metabolism of iron by the Archaea, the purpose of this study was to investigate ferric iron reduction in the Archaeon *A. fulgidus. A. fulgidus* is an anaerobic, sulfate-reducing Archaeon first isolated from marine thermal vents in Southern Italy (18). In this paper, we describe the purification and characterization of a ferric reductase from this extremely thermophilic Archaeon. The *A. fulgidus* enzyme is the first reported ferric reductase isolated from an Archaeon.

EXPERIMENTAL PROCEDURES

Cell Growth Conditions—*A. fulgidus* VC-16 (DSM 4304) was grown anaerobically with 10 mM sodium lactate and 30 mM sodium sulfate at 83 °C. The medium was prepared according to published methods (18) with the following modifications: the carbonate buffer system was replaced by 20 mM PIPES,[1] pH 7.0 (pH adjusted at room temperature), and sodium acetate was omitted. Large scale cultures were grown in 70-liter batches using a custom-built 100-liter glass-lined steel fermentor (Pfaudler, Rochester, NY). The cells were harvested in late log phase (24 h after inoculation) at an optical density (OD_{660}) of 0.5–0.6 by concentrating with an A/G Technology hollow fiber unit (Needham, MA,

* This work was supported by National Institutes of Health Grant HL-16251 and National Sciennce Foundation Grant MCB-9631006 (to I. S.) and a grant from the United States Department of Commerce/NIST Cooperative Research Agreement No. 70NANB7H0009 (to H. G. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

nominal molecular weight cut-off 500,000). The concentrated cells were then pelleted by centrifugation for 20 min at 16,000 × *g*. Cell yields were approximately 80 g wet weight per 70 liters of culture.

Purification of Ferric Reductase—All purification procedures were performed aerobically at room temperature unless indicated otherwise. Frozen cells were resuspended in 150 ml of 50 mM Tricine/KOH, pH 7.5, 2 mM $MgCl_2$, 2 mg DNase II. The cells were lysed by passing the cell suspension twice through a French pressure cell (Amicon) at 10,000 pounds/square inch. The insoluble fraction was removed by centrifugation at 130,000 × *g* for 90 min. A part of the soluble fraction (10 ml, 180 mg of protein) was loaded onto a 5-ml Q-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 20 mM PIPES, pH 7.0. The column was washed with 25 ml of PIPES buffer containing 10 mM NaCl, and ferric reductase was eluted with PIPES buffer containing 60 mM NaCl. The eluted protein was applied to a 5-ml Red-agarose affinity column (Sigma) equilibrated with 20 mM PIPES, pH 7.0. After washing the column with 25 ml of the PIPES buffer containing 0.7 M NaCl, the ferric reductase was eluted with 0.9 M NaCl in PIPES buffer. Ammonium sulfate was added to the eluent to a concentration of 1.7 M, and the proteins were applied onto a 1-ml butyl-Sepharose hydrophobic interaction column (Amersham Pharmacia Biotech) equilibrated with PIPES buffer containing 1.7 M $(NH_4)_2SO_4$. The column was washed with buffer containing 1.25 M $(NH_4)_2SO_4$, and protein containing ferric reductase activity was eluted with buffer containing 1.15 M $(NH_4)_2SO_4$. The purified protein was stored either at 4 °C or at −20 °C for long term.

Ferric Reductase Assay—The ferric reductase assay was adapted from the procedure described by Berczi *et al.* (6). The assay was performed anaerobically in stoppered quartz cuvettes at 85 °C. The assay mixture contained 50 mM sodium phosphate buffer, pH 7.0, 0.25 mM Fe^{3+}-EDTA, 5 μM flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), and 0.18–36 μg of protein. The reaction was initiated by the addition of NADH or NADPH to a final concentration of 0.1 mM, and the oxidation of NADH or NADPH was monitored at 340 nm using a DU 640 spectrophotometer equipped with a high performance temperature controller (Beckman). Electron acceptors other than Fe^{3+}-EDTA were used at a concentration of 0.25 mM. NAD(P)H:flavin oxidoreductase activity was measured with 50 μM FMN or FAD, and Fe^{3+}-EDTA was omitted. One unit of activity is defined as 1 μmol of NAD(P)H oxidized per min.

Characterization of the pH Optimum and Stability—The pH optimum of the ferric reductase enzyme was determined using the following buffers: 50 mM piperazine at pH 6.0, 6.5, and 6.7, 50 mM sodium phosphate at pH 6.8, 7.0, 7.2, 7.4, and 50 mM Tricine at pH 7.9. The pH stability of ferric reductase was determined by incubating the enzyme at room temperature in the buffers listed above. Samples were taken after 1 and 24 h, and ferric reductase activity was determined.

Determination of the Temperature Optimum and Stability—The temperature optimum was determined by performing the ferric reductase assay at the indicated temperatures. The temperature stability of the purified enzyme was determined by incubating the enzyme in stoppered serum vials at the indicated temperatures. Samples were removed at the indicated times, and the activity was measured at 85 °C.

Protein Determination—The protein concentration was measured with the Bradford assay (Bio-Rad) using bovine serum albumin as the standard.

Gel Electrophoresis—Proteins were separated on 20% homogenous polyacrylamide gels under denaturing conditions with the Amersham Pharmacia Biotech Phastsystem. Prior to loading, the protein samples were incubated in SDS gel-loading buffer for 30 min at 100 °C (19). Precast SDS-polyacrylamide gels were purchased from Amersham Pharmacia Biotech.

Molecular Weight Determination—The apparent molecular weight of the purified, denatured ferric reductase was determined from the mobility of the protein in SDS-PAGE. The molecular weight standards were bovine albumin (M_r 66,000), chicken egg albumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), bovine carbonic anhydrase (M_r 29,000), bovine pancreas trypsinogen (M_r 24,000), soybean trypsin inhibitor (M_r 20,000), and bovine milk α-lactalbumin (M_r 14, 200) (Sigma). The apparent molecular weight of the native ferric reductase was determined by size exclusion chromatography with a Superose 6 column from Amersham Pharmacia Biotech. The column was run in 50 mM PIPES, pH 7.0, 200 mM NaCl at a flow rate of 0.4 ml/min. The following molecular weight markers from Amersham Pharmacia Biotech were used: ribonuclease A (M_r 13, 700), chymotrypsinogen A (M_r 25,000), ovalbumin (M_r 43,000), and bovine serum albumin (M_r 67,000).

N-terminal Amino Acid Sequence Analysis—The purified protein was run on a 20% polyacrylamide SDS gel and transferred to a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad). The N-terminal sequence analysis was performed by the Protein Microsequencing Facility at UCLA.

Redox Titration of Complexed Fe^{3+}—The redox potentials of Fe^{3+}-EDTA, Fe^{3+}-NTA, and Fe^{3+}-Citrate were determined with an Omni 90 potentiostat set to cycle the voltage at 50 mV/s (Cypress Systems Inc.). The potentiostat was equipped with an Ag/$AgCl_2$ reference electrode, a platinum auxiliary electrode, and a gold working electrode. Voltammetry was were performed under anaerobic conditions in 20 mM PIPES, pH 7.0. The Fe^{3+} complexes were at a concentration of 0.4 mM.

TABLE I

Purification of the ferric reductase from A. fulgidus

The activity was determined at 85 °C with NADPH as electron donor, Fe^{3+}-EDTA as the acceptor, and 5 μM FMN as electron mediator.

Preparation	Protein	Units[a]	Recovery	Specific activity	Purification
	mg		*%*	*units[a]/mg*	*-fold*
Soluble fraction	180.0	3600	100	20	1
Q-Sepharose	15.18	3324	92.3	219	11
Red-agarose	0.97	928	25.7	959	48
Butyl-Sepharose	0.22	591	16.4	3503	175

[a] Unit is defined as micromoles of NADPH oxidized per min.

RESULTS

Ferric Reductase Activity—By using a modified ferric reductase assay adapted to 85 °C, high ferric reductase activity was detected in the soluble protein fraction of *A. fulgidus* VC-16 grown on lactate as carbon and energy source and sulfate as the electron acceptor. With NADPH as the electron donor and Fe^{3+}-EDTA as the electron acceptor, the enzyme activity in the soluble fraction was determined to be 1 unit/mg protein. Addition of synthetic FMN to the assay stimulated the activity in the soluble fraction by 20-fold (*i.e.* 20 units/mg protein) (Table I). In contrast, the purified protein strictly required FMN as a catalytic intermediate for activity (see below). All assay components were stable at 85 °C for the duration of the assay (at least 2 min). The membrane fraction did not contain any measurable ferric reductase activity (<0.05 units/mg). Cell extract prepared from the hyperthermophilic Archaeon, *Pyrobaculum aerophilum*, did not exhibit ferric reductase activity (data not shown).

Purification of the Ferric Reductase—Ferric reductase was purified to homogeneity using Q-Sepharose ion exchange, Red-agarose affinity, and butyl-Sepharose hydrophobic interaction chromatography (Fig. 1 and Table I). After the final purification step, the enzyme had been enriched 175-fold with a yield of 16%. Based on this yield, ferric reductase was calculated to comprise about 0.75% of the total soluble *A. fulgidus* protein (w/w). By using SDS-PAGE, it was determined that the purified ferric reductase consists of a single polypeptide with an apparent molecular weight of 18,000 (Fig. 1). The molecular weight of the native enzyme was determined to be 40,000 by size exclusion chromatography, suggesting that ferric reductase exists as a homodimer (data not shown). After purification, ferric reductase did not display any significant absorption in the visible range indicating that chromophores such as flavin, heme, and/or Fe-S centers were absent (data not shown).

Flavin Dependence of Ferric Reductase—During protein purification, it was noted that ferric reductase activity was gradually lost. When the soluble *A. fulgidus* protein was subjected to a desalting column, the activity was almost abolished suggesting that a low molecular weight compound was required to retain activity. A yellow-colored fraction that had been eluted from the desalting column in the column volume restored ferric reductase activity. This yellow compound exhibited a visible absorption spectrum characteristic of flavins (data not shown). The preparation was not further characterized. Synthetic FMN, FAD, but not riboflavin, also restored ferric reductase activity (Table II, data for FAD and riboflavin not shown).

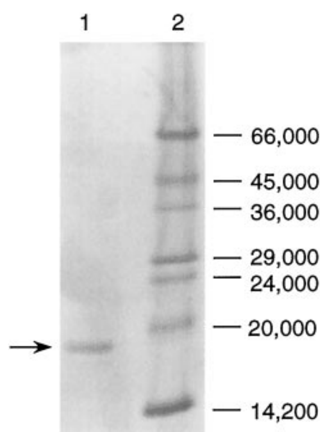


FIG. 1. **SDS-PAGE analysis of the purified *A. fulgidus* ferric reductase.** *Lane 1*, 1 μg of ferric reductase; *lane 2*, molecular weight markers (see under "Experimental Procedures").

TABLE II
FMN dependence of purified ferric reductase activity from A. fulgidus determined with NADPH as the electron donor

Additions to enzyme assay			Specific activity
Fe^{3+}-EDTA	Enzyme	FMN	
mM	*μg*	*μM*	*%*
0.25	0.2	5	100
0.25	0.2	0	<1
0	0.2	50	8
0.25	0	5	<1

Without added flavin, no activity was observed demonstrating that purified ferric reduction was strictly dependent on either FMN or FAD.

To examine whether flavin could serve as the electron acceptor in place of Fe^{3+}-EDTA, activity of the purified enzyme was measured in the absence of Fe^{3+}-EDTA using a 10-fold higher concentration of FMN, FAD, or riboflavin than in the ferric reductase assay (Table II and Table III). Both FMN and FAD, but not riboflavin, served as electron acceptors with NADH or NADPH as electron donors. Therefore, the ferric reductase also functions as an NAD(P)H:flavin oxidoreductase similar to the ferric reductases of *Saccharomyces cerevisiae* and *Escherichia coli* (7, 20–22). Fig. 2*A* shows the reduction of FAD by ferric reductase with NADH as the electron donor. In contrast, FAD remains oxidized in the presence of Fe^{3+}-EDTA consistent with the notion that FAD serves here as an electron mediator (Fig. 2*B*). Unlike many bacterial ferric reductases, the *A. fulgidus* enzyme does not require Mg^{2+} for optimal activity (data not shown) (2, 3, 23).

Since the *A. fulgidus* ferric reductase lacked any associated flavin, a reconstitution of the enzyme with flavin was attempted. Ferric reductase was incubated with 1 mM FMN for 10 min at 60 °C. After incubation, the enzyme was subjected to a desalting and subsequently to an ion exchange chromatography column (*i.e.* Q-Sepharose). Whereas free FMN eluted in the total volume of the desalting column and did not bind to the Q-Sepharose column, the ferric reductase enzyme emerged brightly yellow-colored from the void volume of the desalting and the NaCl eluent of the Q-Sepharose column. This result demonstrates that the ferric reductase could be reconstituted with FMN. The FMN is, therefore, non-covalently bound to the enzyme. Based on the extinction coefficient for free FMN (12.2 mM^{-1} cm^{-1} at 450 nm) the amount of FMN per monomer was determined to be 1.4 mol/mol.

Kinetic Properties of the Ferric Reductase—The kinetic properties of the ferric reductase were determined at 85 °C and are summarized in Table III. With Fe^{3+}-EDTA as the electron acceptor, the purified ferric reductase exhibited a slightly higher affinity and V_{max} for NADH than for NADPH as the electron donor. Based on the molecular weight of the dimeric ferric reductase deduced from the gene sequence (*i.e.* 37, 318, see below) turnover numbers of 3069 and 2180 s^{-1} for NADH and NADPH oxidation, respectively, were calculated. The K_m for FMN as catalytic intermediate in ferric iron reduction and as electron acceptor in place of Fe^{3+}-EDTA were identical (data not shown) and more than 200-fold lower than that for Fe^{3+}-EDTA (Table III). The V_{max} values with FMN and FAD were about 12-fold slower than that determined with Fe^{3+}-EDTA.

TABLE III
Kinetic properties of the ferric reductase from A. fulgidus

Assay components		V_{max}	K_m	
e-donor	e-acceptor		e-donor	e-acceptor
		units/mg	*μM*	
NADH	Fe^{3+}-EDTA	4935	61	ND[a]
NADPH	Fe^{3+}-EDTA	3505	80	66
NADPH	FMN[b]	280	ND[a]	0.3
NADPH	FAD	350	ND[a]	ND[a]
NADPH	Riboflavin	<0.1	ND[a]	ND[a]

[a] ND, not determined.
[b] The FMN concentration was 50 μM.

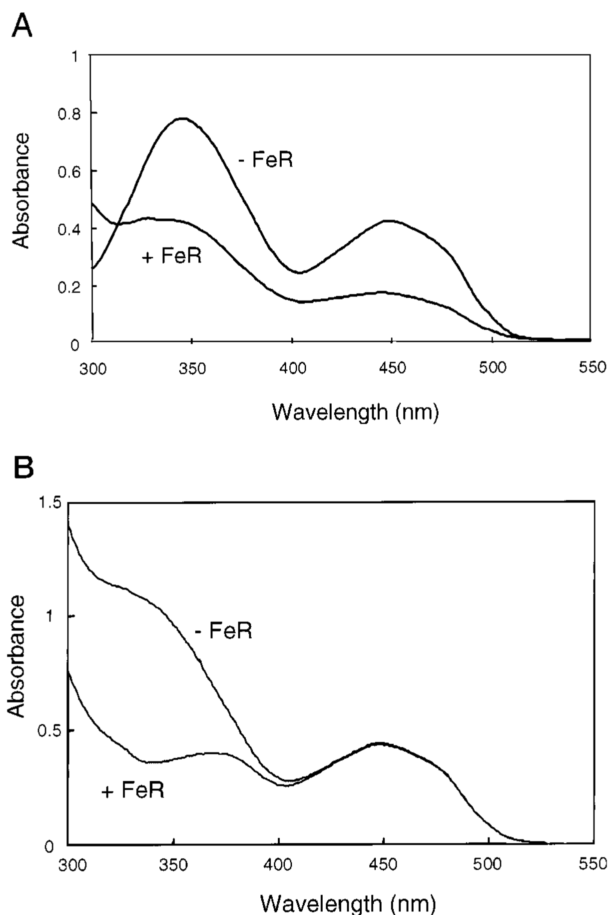


FIG. 2. **Absorption spectrum of FAD reduction with NADH by purified ferric reductase (*FeR*) in the absence (*A*) and presence of 200 μM Fe^{3+}-EDTA (*B*).** The reaction buffer contained 50 mM NaH_2PO_4-Na_2HPO_4, pH 7.0, 100 μM NADH, and 40 μM FAD. Spectra were recorded after anaerobic incubation for 5 min at 80 °C without (−*FeR*) and with ferric reductase (0.25 μg) (+*FeR*).

TABLE IV
Substrate specificity for various electron acceptors of the ferric reductase from A. fulgidus

The activities were measured with NADPH as the electron donor and 5 μM FMN as electron mediator. ND, not determined; DCPIP, dichlorophenolindophenol; DMN, dimethylmaphtoquinone.

Electron acceptor[a]	E_0' (V)	Specific activity[b]
		%
Fe^{3+}-EDTA	0.104[c]	100
Fe^{3+}-NTA	0.144[c]	12
Fe^{3+}-citrate	0.146[c]	6
Fe^{2+}-EDTA	ND	<1
Ferricyanide	0.36	252
DCPIP	0.217	58
DMN	−0.08	<1

[a] The electron acceptors were 0.25 mM final concentration.
[b] The activity was determined with NADPH as the electron donor and 5 μM FMN present.
[c] The redox potential of the Fe^{3+} complexes is given as E' as it was determined, pH 7.0, and at 0.4 mM concentrations (see "Experimental Procedures").

The corresponding turnover numbers were 174 s^{-1} for FMN reduction and 216 s^{-1} for FAD reduction.

Electron Acceptors of Ferric Reductase—Ferric reductase also exhibited activity with other complexes that contained Fe^{3+} as the electron acceptor (Table IV). The highest activity was obtained with sodium ferricyanide; it was 2.5 times higher than the activity measured with Fe^{3+}-EDTA. Activities of 12% or less were obtained with other ferric complexes such as Fe^{3+}-NTA and Fe^{3+}-citrate. With dichlorophenolindophenol, an artificial electron acceptor of many flavoproteins, ferric reductase activity was half that with Fe^{3+}-EDTA. All electron acceptors were reduced only when catalytic amounts of FMN were present. No activity was obtained with uncomplexed Fe^{3+}, *i.e.* $FeCl_3$ and $Fe(OH)_3$ (data not shown), with the menaquinone analog dimethylnaphthoquinone or with Fe^{2+}-EDTA (Table IV). The latter compound served as a control to demonstrate that the Fe^{3+} and not the EDTA part of the complex was reduced by the enzyme. Finally, ferric reductase did not reduce EDTA-chelated Ag^{+} and Cu^{2+} (data not shown).

pH and Temperature Optima and Stability of Ferric Reductase—The *A. fulgidus* ferric reductase exhibited a pH optimum of 7.0 (Fig. 3). To ensure that the decrease of enzyme activity seen below and above pH 7.0 was not due to enzyme instability, ferric reductase was incubated in buffers at the different pH values for 24 h. The enzyme activity was unchanged under all conditions suggesting that the loss of activity below and above the pH optimum is due to protonation and deprotonation of active site residues.

Ferric reductase activity was highest at 88 °C (Fig. 4). This temperature is close to the optimal growth temperature of *A. fulgidus* (*i.e.* 83 °C). To evaluate the enzyme's temperature stability, ferric reductase was incubated at various temperatures, and its activity was measured at 85 °C (Fig. 5). The enzyme was highly stable at room temperature retaining more than 90% of its activity over 4 weeks, but it denatured with a half-life of 4 min when incubated at 100 °C. At incubation temperatures of 65 and 85 °C the half-life was 6.5 and 2 h, respectively.

Amino Acid Sequence Analysis of the Ferric Reductase—The 19 N-terminal amino acids of the *A. fulgidus* ferric reductase were determined to be Met-Asp-Val-Glu-Ala-Phe-Tyr-Lys-Ile-Ser-Tyr-Gly-Leu-Tyr-Ile-Val-Thr-Ser-Glu. The amino acid sequence was used to search the *A. fulgidus* genomic sequence data base of The Institute for Genomic Research. A 100% match was found to a hypothetical protein encoded by the predicted coding region AF0830 (24). The hypothetical protein contains 169 amino acids, which corresponds to a molecular weight of 18,659. This molecular weight is consistent with that of the purified enzyme as determined by SDS-PAGE (Fig. 1).

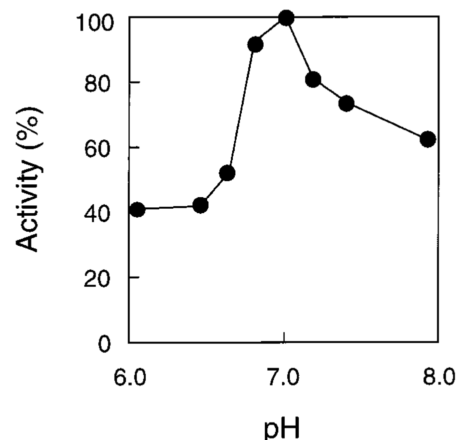


FIG. 3. **pH dependence of the *A. fulgidus* ferric reductase activity.** The activity was measured as described under "Experimental Procedures."

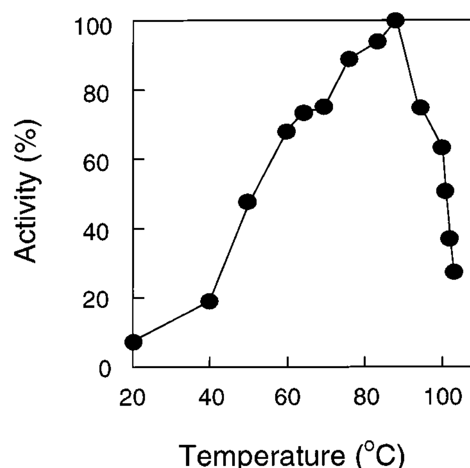


FIG. 4. **Temperature dependence of the *A. fulgidus* ferric reductase activity.** The activity was measured at 85 °C as described under "Experimental Procedures."

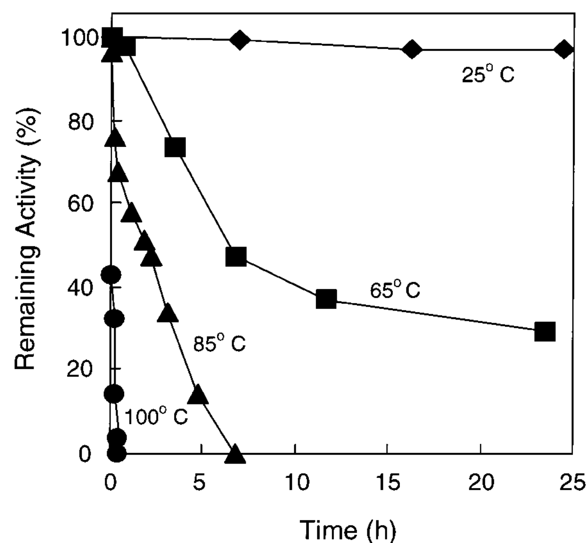


FIG. 5. **Thermal stability of the *A. fulgidus* ferric reductase.** The enzyme was incubated at the temperatures indicated in the figure. At various times samples were withdrawn, and ferric reductase activity was measured at 85 °C as described under "Experimental Procedures."

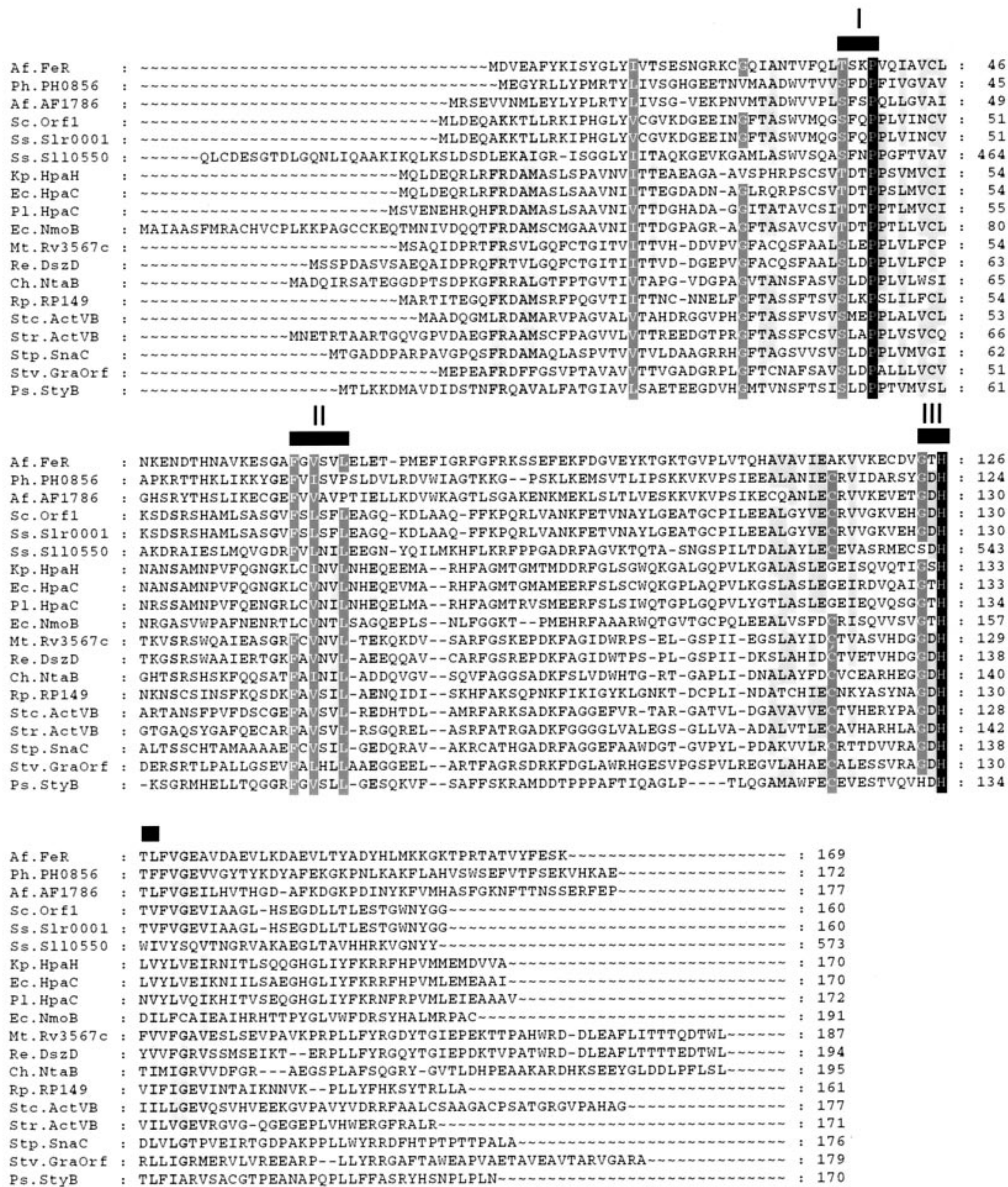


FIG. 6. **Multiple sequence alignment of *A. fulgidus* ferric reductase-related proteins generated by pileup and edited by GeneDoc.** Consensus residues are indicated by *white letters on black background* for 100% homology and *white letters on gray background* for 80% homology. *Bars above* the sequence indicated the location of the conserved regions I, II, and III. Abbreviations and accession numbers for proteins are as follows: Af.FeR, *A. fulgidus* ferric reductase (gi 2649784); Ph.Ph0856, *Pyrococcus horikoshii* hypothetical protein (gi 3257267); Af.AF1786, *A. fulgidus* hypothetical protein (gi 2648759); Sc.Orf1, *Synechococcus* sp. (strain PCC 7942) hypothetical protein (gi 310858); Ss.Slr0001, *Synechocystis* sp. (strain PCC 6803) hypothetical protein (gi 1001553); Ss.Sll0550, *Synechocystis* sp. (strain PCC 6803) putative flavodoxin (gi 1001242); Kp.HpaH, *K. pneumoniae* coupling protein (gi 974147); Ec.HpaC, *E. coli* component C of the 4- hydroxyphenylacetate-hydroxylase (gi 757835); Pl.HpaC, *Photorhabdus luminescens* 4-hydroxyphenylacetic acid hydroxylase putative coupling protein (gi 3002549); Ec.NmoB, *E. coli* 4-hydroxyphenylacetate 3-monooxygenase small chain (gi 4062570); Mt.Rv3567c, *M. tuberculosis* hypothetical nitrilotriacetate monooxygenase component B (gi 1877298); Re.DszD, *Rhodococcus erythropolis* NADH:FMN oxidoreductase (gi 2944380); Ch.NtaB, *C. heintzii* nitrilotriacetate monooxygenase component B (gi 2507085); Rp.RP149, *Rickettsia prowazekii* hypothetical protein (gi 3860716); Stc.ActVB, *S. coelicolor* actinorhodin polyketide dimerize (gi 2498304); Str.ActVB, *S. roseofulvus* ActVB homolog (gi 3170573); Stp.SnaC, *S. pristinaespiralis* NADH:FMN oxidoreductase (gi 1711412); Stv.GraOrf *Streptomyces violaceoruber* putative FMN:NADH oxidoreductase (gi 4218572); Ps.StyB, *P. fluorescens* StyB protein (gi 2154928).

The gene product of AF0830 is thus identified as a ferric reductase or, alternatively, as a NAD(P)H:flavin oxidoreductase. The corresponding gene is designated *fer* for <u>fe</u>rric <u>r</u>eductase. The enzyme has a theoretical isoelectric point of 5.84.

Various data bases were searched for similar proteins. However, no homology existed to any known ferric reductase. Homology of the *A. fulgidus* ferric reductase was found to members of a family of NAD(P)H:flavin oxidoreductases and to other hypothetical proteins from a variety of prokaryotic organisms (Fig. 6). The NAD(P)H:flavin oxidoreductase family includes the HpaC proteins from *E. coli*, *Photorhabidus luminescence*, *Klebsiella pneumoniae* (here called HpaH), and the *E. coli* NmoB protein all of which are involved in oxidative cleavage of 4-hydroxyphenylacetate (25–27). The C-terminal domain of the *Chelatobacter heintzii* NtaB protein is involved in the hydroxylation of nitrilotriacetate (28–30). Other family members are the *Pseudomonas* sp. StyB protein involved in styrene hydroxylation, as well as the *Streptomyces pristinaespiralis*

SnaC protein and the ActVB proteins from *Streptomyces coelicolor* and *Streptomyces roseofulvus* involved in the synthesis of antibiotics (31–35). The sequence identity between these proteins and the *A. fulgidus* ferric reductase extends to about 26% with a similarity of 47%.

Visual inspection of the amino acid sequences of the *A. fulgidus* ferric reductase and related proteins shown in Fig. 6 revealed 3 short regions that appear to be significantly conserved. These regions are now defined as regions I, II, and III. Region I includes the amino acid residues 36–39, (T/S)*XX*P, of the *A. fulgidus* ferric reductase, where *X* indicates a variable amino acid residue. Regions II and III include amino acids 62–67, F*X*(L/I/V)*X*(L/I/V)L, and amino acids 124–128, G(T/D)H*X*(L/I/V) of the *A. fulgidus* ferric reductase sequence, respectively. None of the regions is homologous to any known flavin- or NAD-binding sites. However, these regions might be involved in flavin and/or NAD coordination.

DISCUSSION

A. fulgidus, when grown on lactate and sulfate, contained the enzyme, ferric reductase, in its soluble protein fraction. The ferric reductase was purified to homogeneity and found to be very abundant in *A. fulgidus* (0.75% of the soluble fraction) suggesting that it may have an important function in the *A. fulgidus* metabolism. The enzyme catalyzes the reduction of Fe^{3+}-EDTA and other Fe^{3+} complexes with NADH or NADPH as the electron donors and requires the addition of free flavin for maximal activity.

The ferric reductase from *A. fulgidus* exhibits its highest activity at 88 °C, which is close to the temperature optimum for growth. Other enzymes isolated *from A. fulgidus*, *i.e.* the isocitrate dehydrogenase, the NADP-specific glutamate dehydrogenase, and the L-malate dehydrogenase exhibit an even higher temperature optimum for activity of 90 °C or greater (36–38). The latter enzymes have been reported to be very thermostable with a half-life of 80 min at 101 °C for the L-malate dehydrogenase, and 140 min at 100 °C for the NADP-dependent glutamate dehydrogenase (37, 38). In contrast, the ferric reductase exhibits a half-life of 4 min at 100 °C and is thus more similar to the *A. fulgidus* isocitrate dehydrogenase (36).

Ferric reductases have been described previously in eukarya and bacteria. Almost all of these enzymes are involved in iron assimilation metabolism. This work presents the first characterization of an archaeal ferric reductase. The *A. fulgidus* ferric reductase lacks any prosthetic groups after purification but strictly requires free FMN or FAD for the reduction of complexed Fe^{3+}. In this respect, the *A. fulgidus* ferric reductase resembles the assimilatory bacterial ferric reductase enzymes isolated from *Rhodobacter sphaeroides*, *Neisseria gonorrhoeae*, and *E. coli* that also require free flavin for ferric reduction. (23, 39, 40). In addition, the *A. fulgidus* enzyme utilizes flavin as a substrate and thus functions also as NAD(P)H:flavin oxidoreductase. Since it was possible to reconstitute the *A. fulgidus* ferric reductase with FMN, this enzyme clearly classifies as a flavoprotein, and we suggest that the enzyme-bound flavin is involved in catalysis. This is substantiated by the very high affinity of the purified enzyme for FMN (*i.e.* 0.3 μM). Therefore, for the reduction of free flavin by NAD(P)H, Reactions 1–6 are postulated as follows:

$$E + \text{FMN} \rightleftarrows E\text{-FMN}$$

REACTION 1

$$E\text{-FMN} + \text{NAD(P)H} \rightleftarrows E\text{-FMN-NAD(P)H} \rightleftarrows E\text{-FMNH}_2 + \text{NAD(P)}$$

REACTION 2

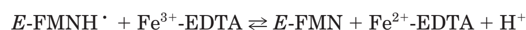
$$E\text{-FMNH}_2 + \text{FMN} \rightleftarrows E\text{-FMN} + \text{FMNH}_2$$

REACTION 3

Since the affinity for FMN is significantly higher (*i.e.* 220-fold) than for Fe^{3+}-EDTA, it is likely that enzyme-bound flavin is also involved in the catalysis of Fe^{3+}-EDTA reduction, which is described by Reactions 4–6.

$$E\text{-FMNH}_2 + \text{Fe}^{3+}\text{-EDTA} \rightleftarrows E\text{-FMNH}^{\cdot} + \text{Fe}^{2+}\text{-EDTA} + \text{H}^+$$

REACTION 4



$$E\text{-FMNH}^{\cdot} + \text{Fe}^{3+}\text{-EDTA} \rightleftarrows E\text{-FMN} + \text{Fe}^{2+}\text{-EDTA} + \text{H}^+$$

REACTION 5

or

$$2E\text{-FMNH}^{\cdot} \rightleftarrows E\text{-FMN} + E\text{-FMNH}_2$$

REACTION 6

The different k_{cat} values for flavin *versus* ferric reduction can be explained by Reaction 3 being rate-limiting for flavin reduction, whereas Reactions 4 and 5 (or 6) are much faster than Reaction 3 and rate-determining for the ferric reductase activity.

Whereas the affinities for NAD(P)H, Fe^{3+} complexes, and FMN among the bacterial ferric reductases and the archaeal enzyme are similar, the *A. fulgidus* enzyme differs strikingly by its very high specific activity. The activity of the *A. fulgidus* enzyme is at least 1000-fold higher than all other bacterial ferric reductase activities that have been measured thus far (Table I), and it is 40 times more active than the fastest eukaryotic ferric reductase isolated from spinach (6). Enzymes from extremeophiles usually exhibit enzyme activities that are in the same range as their mesophilic counter parts. Examples for thermophilic enzymes from *A. fulgidus* that have specific activities like their mesophilic counter parts include the isocitrate, L-malate, and NADP-dependent glutamate dehydrogenases (36, 37, 38).

E. coli contains three distinct enzymes that serve as assimilatory ferric reductases (42). Like the major ferric reductase in *E. coli*, the *A. fulgidus* enzyme also functions as an NAD (P)H: flavin oxidoreductase (see above) (42). However, the *A. fulgidus* enzyme has a specific activity more than 750-fold higher than the major *E. coli* flavin reductase, Fre. Whereas the *A. fulgidus* enzyme also utilizes FAD and FMN as electron acceptors, riboflavin does not serve as a substrate. This is unlike the *E. coli* major NAD(P)H:flavin oxidoreductase, which reduces FMN, FAD, and riboflavin (20). This NAD(P)H:flavin oxidoreductase has been demonstrated to activate ribonucleotide reductase in *E. coli* by reducing the enzyme's [Fe-S] center, which subsequently generates a tyrosyl radical (39). Coves and Fontecave (20) suggested that the reduced flavin produced by the *E. coli* NAD(P)H:flavin oxidoreductase serves to reduce chemically several suitable electron acceptors. It is, therefore, feasible that the *A. fulgidus* ferric reductase may also have a dual function in the metabolism of *A. fulgidus*.

N-terminal amino acid sequence analysis of the purified ferric reductase resulted in the identification of a hypothetical protein in *A. fulgidus* with no previously known function (*i.e.* encoded by gene *AF0830*). This protein is now assigned the function of a ferric reductase and an NAD(P)H:flavin oxidoreductase. Interestingly, no homology was found between the *A. fulgidus* ferric reductase and the ferric reductases from *S. cerevisiae* or from *E. coli* (21, 22). However, significant homology of the *A. fulgidus* ferric reductase exists to a family of NAD(P)H:flavin oxidoreductases that are members of a two-

enzyme system, in which the NAD(P)H:flavin oxidoreductase generates reduced FMN for a monooxygenase (Fig. 6). In general, these monooxygenases are involved in the oxidative degradation of aromatic compounds, hydrocarbons, or in polyketide biosynthesis. Although the deduced amino acid sequence for the *A. fulgidus* ferric reductase did not reveal any known motif conclusive for the coordination of a prosthetic group, three conserved regions were noted (Fig. 6). These regions may be involved in the coordination of flavin and/or NAD. The three regions noted in this study are also present in the C-terminal domains of 4 *Synechocystis* sp. A-type flavoproteins of unknown function (43). The latter proteins contain a partially conserved flavodoxin signature within their N-terminal domain, which is speculated to be responsible for the binding of FMN. One of the *Synechocystis* flavoproteins was demonstrated to bind both FAD and FMN (43); however, it is not known whether this flavoprotein interacts with NADH or Fe^{3+}.

The *A. fulgidus* ferric reductase is distinct from the dissimilatory cytochrome *c*-type metal reductases isolated from the iron-reducing bacteria *D. acetoxidans* and *G. sulfurreducens* (15, 16). These proteins have broad substrate specificity for complexed and uncomplexed Fe^{3+} compounds, manganese dioxide, elemental S^0, and other compounds. In contrast, the *A. fulgidus* ferric reductase does not use uncomplexed iron and has its highest specificity for Fe^{3+}-EDTA, although the redox potentials for Fe^{3+}/Fe^{2+}-NTA and Fe^{3+}/Fe^{2+}-citrate are almost identical to that of Fe^{3+}/Fe^{2+}-EDTA (Table IV).

The physiological role of ferric reductase in the sulfate-reducing *A. fulgidus* is uncertain. Functionally, the enzyme is more similar to the assimilatory ferric reductases. However, because of its high cellular abundance (see above), it seems possible that this enzyme serves a dissimilatory rather than an assimilatory function. Vargas *et al.* (17) previously reported on ferric reductase activity in *A. fulgidus*. In their study, the activity was measured with cell suspensions using hydrogen as the electron donor and Fe^{3+}-citrate as the electron acceptor. The authors concluded that this reaction could potentially be used to generate energy for growth, although growth of *A. fulgidus* with Fe^{3+}-citrate was not demonstrated. The ferric reductase purified in this study could be the key enzyme of this possible electron transfer pathway since the purification yielded only one type of this enzyme. A speculative electron transfer chain could contain a hydrogenase that oxidizes hydrogen to protons on the outside of the cell. The electrons could be transferred across the cytoplasmic membrane to an NADH dehydrogenase that would consume protons on the cytoplasmic side to generate NADH. NADH would then be regenerated by the cytoplasmically located ferric reductase. The scalar proton translocation would be sufficient to generate the proton motive force. This electron transfer pathway resembles somewhat the sulfate reduction pathway, which also involves soluble terminal reductases, *i.e.* the APS reductase and the sulfite reductase (44–46). Whether an electron transport chain involving the ferric reductase characterized in this study exists in *A. fulgidus* still has to be established.

Acknowledgment—We thank the anonymous reviewer of this manuscript for the helpful suggestions regarding the flavin reconstitution and the flavin and ferric reduction mechanism, which greatly strengthened our manuscript.

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