Flavin Mononucleotide-Binding Flavoprotein Family in the Domain *Archaea*

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The protein (AfpA, for archaeoflavoprotein) encoded by AF1518 in the genome of *Archaeoglobus fulgidus* was produced in *Escherichia coli* and characterized. AfpA was found to be a homodimer with a native molecular mass of 43 kDa and containing two noncovalently bound flavin mononucleotides (FMNs). The cell extract of *A. fulgidus* catalyzed the CO-dependent reduction of AfpA that was stimulated by the addition of ferredoxin. Ferredoxin was found to be a direct electron donor to purified AfpA, whereas rubredoxin was unable to substitute. Neither NADH nor NADPH was an electron donor. Ferricyanide, 2,6-dichlorophenolindophenol, several quinones, ferric citrate, bovine cytochrome *c*, and O₂ accepted electrons from reduced AfpA, whereas coenzyme F₄₂₀ did not. The rate of cytochrome *c* reduction was enhanced in the presence of O₂ suggesting that superoxide is a product of the interaction of reduced AfpA with O₂. Although AF1518 was previously annotated as encoding a decarboxylase involved in coenzyme A biosynthesis, the results establish that AfpA is an electron carrier protein with ferredoxin as the physiological electron donor. The genomes of several diverse *Archaea* contained *afpA* homologs clustered with open reading frames annotated as homologs of genes encoding reductases involved in the oxidative stress response of anaerobes from the domain *Bacteria*. A potential role for AfpA in coupling electron flow from ferredoxin to the putative reductases is discussed. A search of the databases suggests that AfpA is the prototype of a previously unrecognized flavoprotein family unique to the domain *Archaea* for which the name archaeoflavoprotein is proposed.

Archaeoglobus fulgidus strain VC-16 is a strictly anaerobic hyperthermophile that obtains energy for growth by oxidizing lactate and reducing sulfate to sulfide (28). *Archaeoglobus* is the only known dissimilatory sulfate-reducing genus classified in the domain *Archaea* and is closely related to the methane-producing genus *Methanosarcina* in the order *Methanosarcinales* of the domain *Archaea* (1). In *A. fulgidus*, lactate is oxidized to pyruvate and then acetyl-coenzyme A. The acetyl-coenzyme A is cleaved into methyl and carbonyl groups that are further oxidized to CO₂ involving enzymes and cofactors of energy-yielding pathways also operative in *Methanosarcina* species (21, 22). CO-dehydrogenase/acetyl-coenzyme A synthase (CODH/ACS) cleaves acetyl-coenzyme A and oxidizes the carbonyl group to CO₂ during growth of *A. fulgidus* on lactate (22) and *Methanosarcina* species on acetate (13). Ferredoxin is the electron acceptor for the CODH/ACS from both *A. fulgidus* (10) and *Methanosarcina* species (12). Coenzyme F₄₂₀ is the electron acceptor for steps in oxidation of the methyl group of acetyl-coenzyme A to CO₂ in *A. fulgidus* except the last step, for which the electron acceptor is unknown.

Isf (for iron-sulfur flavoprotein) is an electron transfer protein first discovered in *Methanosarcina thermophila* and is the prototype of a family which includes homologs found in diverse anaerobic species representing all three domains (32). A striking feature of the family is the almost universal occurrence, in individual species, of multiple *isf* homologs. The genomic se-

quence of *A. fulgidus* strain VC-16 reveals three *isf* homologs. The *isf-2* (AF1519) homolog is one of three tightly clustered genes oriented in the same direction, consistent with an operon (32). Remarkably, an *isf* homolog (MJ0731) in the genomic sequence of *Methanocaldococcus jannaschii* (*Methanococcus jannaschii*) is closely flanked by MJ0732 and MJ0730 with deduced sequences having 46 and 50% identity to the putative proteins encoded by AF1520 and AF1518 that flank *isf-2* in *A. fulgidus* (32). The *M. jannaschii* open reading frames (ORFs) MJ0732-MJ0731-MJ0730 are all oriented in the same direction. *M. jannaschii* is a methane-producing anaerobe from the order *Methanococcales* in the domain *Archaea*. The conserved arrangement of genes in *A. fulgidus* and *M. jannaschii* with corresponding high deduced sequence identity (AF1520-*isf*-AF1518 and MJ0732-*isf*-MJ0730) is consistent with related physiological functions among the gene products.

Here we present the properties of the AF1518 gene product (AfpA, archaeoflavoprotein) which show that AfpA is a flavin mononucleotide (FMN)-containing electron carrier protein linked to the CODH/ACS complex through ferredoxin. Furthermore, AfpA was shown to be the prototype of a previously unrecognized family of FMN-containing flavoproteins identified only in *A. fulgidus* and methane-producing species from the domain *Archaea*. A role for AfpA and the products of genes clustered with *afpA* in the oxidative stress response operations of members of the domain *Archaea* is discussed.

MATERIALS AND METHODS

Materials. The 5- and 6-glutamylated homologs of coenzyme F₄₂₀ were a gift from Lacey Daniels, University of Iowa. 2-Hydroxyphenazine was a gift from Uwe Deppenmeier, Georg-August-Universität. *A. fulgidus* genomic DNA and *Pyrococcus furiosus* rubredoxin were gifts from Michael Adams and Francois

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Jenney, University of Georgia. All other enzymes and reagents were purchased from Sigma (St. Louis, Mo.). *A. fulgidus* cells grown on lactate were a gift from Robert Kelly, North Carolina State University. *M. jannaschii* JAL-1 cells grown autotrophically were a gift from Biswarup Mukhopadhyay, Virginia Polytechnic Institute and State University. *M. thermophila* was grown with acetate as described (27). Extracts of cells were prepared as described (32).

Cloning, overexpression, purification, and reconstitution. The full-length AF1518 ORF was amplified by PCR from *A. fulgidus* genomic DNA with sense (ATGTTTGAAATGGAGGAAAAG) and antisense (AGATTTTCCGAAGT GTTTTC) primers (Integrated DNA Technologies, Coralville, Iowa). The initial PCR product was amplified and cloned into the pETBlue-1 AccepTor vector (Novagen, Madison, Wis.) to generate the recombinant expression plasmid pET1518. The AF1518 ORF contains rare codons; thus, pET1518 was transformed into *Escherichia coli* Rosetta (DE3)/pLacI competent cells (Novagen) for heterologous expression. The transformed cells were cultured at 37°C in Luria-Bertani broth containing 100 mg of ampicillin per liter. When the A_{600} reached 0.6, the culture was induced to produce AfpA by the addition of 1 mM IPTG (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) for 16 h at 21°C. Cells were harvested by centrifugation and stored at -80°C.

All steps in the purification and reconstitution procedures were carried out anaerobically with general procedures as previously described (23) and at 21°C except where indicated. Where possible, an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) was used. Thawed cells (30 g wet weight) were resuspended in 120 ml of 50 mM HEPES (pH 7.2) and lysed by passage twice through a French pressure cell at 110 MPa. Cell debris was removed by centrifugation at $74,400 \times g$ for 20 min at 4°C, and the supernatant solution was heated at 70°C for 20 min. Denatured proteins were pelleted by centrifugation at $74,400 \times g$ for 20 min at 4°C, and the supernatant solution filtered through a 0.4- μ m filter. The filtrate was loaded onto a 5-ml cation exchange HiTrap SP column (Amersham Biosciences, Piscataway, N.J.) equilibrated with 50 mM HEPES (pH 7.2). The column was developed with 100 ml of a 0.0 to 1.0 M NaCl linear gradient applied at 2 ml/min. The fractions showing a bright yellow color eluting between 0.2 and 0.25 M NaCl were pooled, concentrated at 4°C by ultrafiltration with a Microcon YM10 membrane (Millipore, Bedford, Mass.), and desalted with a PD-10 column (Amersham Biosciences AB, Uppsala, Sweden). The purified AfpA was reconstituted with FMN as described previously (32) except that 50 mM HEPES (pH 7.2) was used.

Analyses. BLAST searches (4) were performed at www.ncbi.nlm.nih.gov and www.jgi.doe.gov/JGI_microbial/html/index.html. Annotations of finished genomes were obtained at www.tigr.org. Protein concentrations were determined with the bicinchoninic acid assay (26) with chymotrypsinogen A as the standard (Pierce, Rockford, Ill.). The subunit molecular mass was estimated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE) (16) with low-molecular-weight protein standards (Sigma, St. Louis, Mo.). The native molecular mass was determined by size exclusion chromatography with a calibrated Superose-12 gel filtration column (Amersham Biosciences, Piscataway, N.J.) developed with 50 mM HEPES (pH 7.0) containing 200 mM NaCl. The native molecular mass was also determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with a PerSeptive Voyager DE-RP (PerSeptive BioSystems, Framingham, Mass.) instrument at the Pennsylvania State University Mass Spectrometry Center. The matrix was a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid.

The N-terminal amino acid sequence was determined with a model 477A protein sequencer (PE Biosystems, Inc., Foster City, Calif.) at the Macro Facility of the Pennsylvania State University Hershey Medical Center. The UV-visible absorbance spectra were recorded with a Beckman DU7400 diode array spectrophotometer equipped with a circulating water bath for temperature control. Flavin extraction, identification, and quantification were performed as described previously (17).

Spectrophotometric assays. Reduced AfpA reacted rapidly with O_2 necessitating strict anaerobic conditions under an N_2 gas phase, except when O_2 was the electron acceptor. The oxidation of NADH or NADPH ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) by AfpA was determined with either O_2 or $K_3\text{Fe}(\text{CN})_6$ as the electron acceptor. The assay mixture (0.5 ml) contained 50 mM HEPES (pH 7.2, at 21°C and 70°C), 0.2 mM NADPH plus H^+ or NADH plus H^+ , 0.25 mM $K_3\text{Fe}(\text{CN})_6$ or air-saturated buffer. The reaction was initiated with 1.0 or 10.0 μ g of AfpA.

The CO-dependent reduction of AfpA catalyzed by cell extracts was performed as described (17) with the following modifications. The reaction mixture (0.5 ml, 50°C) contained 200 μ g of cell extract protein in 50 mM HEPES (pH 7.2) equilibrated with 1.0 atm of either CO or N_2 . Where indicated, ferredoxin from *Clostridium pasteurianum* was added to the *A. fulgidus* cell extract. The reaction was initiated by the addition of 200 μ g of AfpA and monitored at 457 nm. Assays

in which the headspace gas was N_2 showed no detectable reduction of AfpA. CO dehydrogenase activity was assayed as previously described (30). A coupled enzyme assay was used to determine if ferredoxin or rubredoxin acted as electron donors to AfpA as previously described (9) with the following modifications. The reaction mixture (0.5 ml, 40°C) contained 20 μ g of spinach ferredoxin-NADPH oxidoreductase, *C. pasteurianum* ferredoxin, or *P. furiosus* rubredoxin at the final concentrations indicated, and 0.2 mM NADPH plus H^+ in 50 mM HEPES (pH 7.2). The reaction was initiated by the addition of 200 μ g of AfpA and monitored at 457 nm. No detectable reduction of AfpA occurred in the absence of ferredoxin or rubredoxin.

The ability of AfpA to donate electrons to electron carriers was determined by generating reduced AfpA in the ferredoxin-NADPH oxidoreductase-coupled assay as described above except that spinach ferredoxin was used in place of *C. pasteurianum* ferredoxin. The reaction mixture was heated at 80°C for 5 min to inactivate ferredoxin and ferredoxin-NADPH oxidoreductase. The reaction was initiated by addition of the electron carrier and monitored by following the oxidation of reduced AfpA at 457 nm for each carrier except 2,6-dichlorophenolindophenol ($\epsilon_{600} = 20.0 \text{ mM}^{-1} \text{ cm}^{-1}$) and cytochrome *c* ($\epsilon_{550} = 22.6 \text{ mM}^{-1} \text{ cm}^{-1}$), for which reduction was monitored at 600 and 550 nm, respectively. The assays were determined at 70°C except for the reduction of cytochrome *c*, which was assayed at 21°C.

RESULTS

Analysis of sequences with identity to AfpA. For reasons described below, we propose *afpA* (archaeoflavoprotein) as the gene designation for AF1518. A BLAST search of all nonredundant databases, which included finished and unfinished genomes, was conducted with the entire amino acid sequence deduced from *afpA* as the query. Figure 1A shows alignments of AfpA with the first five sequences having the highest identity to AfpA that were returned by the BLAST search. The total residues for each of the sequences closely approximated that for AfpA and ranged from 44 to 54% identity to AfpA. A total of 50 residues were identified that are strictly conserved and uniformly distributed in all the sequences. The five sequences returned by the BLAST search were annotated as either a conserved protein, a hypothetical protein, or an unspecified flavoprotein. Except for the six sequences shown in Fig. 1B, none of the remaining 94 sequences returned by the search had greater than 20% overall identity with AfpA.

The AF1518 ORF is annotated as encoding both a conserved hypothetical protein and a homolog of *dfp* encoding an enzyme (DNA-pantothenate flavoprotein) in the pathway of coenzyme A biosynthesis catalyzing the decarboxylation of 4'-phosphopantothenate to 4'-phosphopantotheine (14). The BLAST search showed the deduced sequence of AF1518 with significant identity to regions in the N-terminal domains of Dfp enzymes; however, Dfp is typically greater than 400 residues in length compared to the 192 residues for AfpA. Indeed, AF1645 of *A. fulgidus* is annotated as Dfp and the deduced sequence is 404 residues, consistent with a valid annotation for AF1645 and an invalid annotation for AF1518. Furthermore, absent from the sequences in Fig. 1A is the catalytically essential motif (PXMNXXMW) and the substrate recognition clamp motif (P/TX₆C/G/EX₃G/AXG) present in the N-terminal domain of Dfp and other enzymes in the homo-oligomeric flavin-containing cysteine decarboxylase (HFCD) superfamily (14). On the other hand, the sequences in Fig. 1A all have the distinctive motifs (bold residues) which bind FMN in the HFCD superfamily (6). The FMN binding site in the HFCD superfamily is unique among flavoproteins in that the flavin is sandwiched between subunits and the *re* side of the isoalloxazine ring is exposed to solvent (3, 6). This novel FMN binding

FIG. 1. Protein sequence alignments. (A) AfpA and homologs. Bold residues indicate motifs which bind FMN in the HFCD family. (B) AfpA and homologs containing an insert relative to AfpA. Bold residues indicate motifs which bind FMN in the HFCD family and CX₂CX₂CX₃₋₅CP motifs. Sequences were aligned with Clustal X version 1.8 with the default parameters. Values in parentheses indicate percent identity (*) and similarity (:). Organisms and ORF designation from the corresponding genomic sequence are: AF1518, *A. fulgidus* strain VC-16; MA3741 and MA0696, *M. acetivorans* strain C2A; METH3186 and METH2675, *M. barkeri*; MJ0730 and MJ0208, *M. jannaschii*; MM0635 and MM1854, *M. mazei* strain G6; MTH697 and MTH1379, *M. thermoautotrophicus* strain ΔH; MK1484, *M. kandleri* strain AV19.

Figure 1B shows the next six sequences with the greatest identity to AfpA that were returned by the BLAST search. These sequences were found to share between 28 and 37% overall identity with AfpA and to share the same features described above for AfpA except for an insert of 52 to 56 residues containing two CX₂CX₂CX₃₋₅CP sequences characteristic of 4Fe-4S binding motifs (5). All of the returned sequences in Fig. 1B were annotated as either a hypothetical protein or an unspecified flavoprotein. Except for the sequences in Fig. 1A, none of the remaining 93 deduced se-

Recently, it was shown that the deduced sequences of AF1520 and MJ0732 cluster with the hemoflavoprotein rubredoxin-oxygen oxidoreductase from *Desulfovibrio gigas* in a phylogenetic tree of rubredoxin-oxygen oxidoreductase orthologs (25). Rubredoxin-oxygen oxidoreductase is the terminal oxidase of a soluble electron transport chain coupling NADH oxidation and the reduction of O₂ to water (8), a postulated mechanism for O₂ detoxification (15, 25).

B

MA0696 (37,57)	-----MSFKSIWAGIT AGAGH FLDRSYQVFKEKQDRDPE-LSVNTYISRAAEVLRMYGL 54
MM1854 (37,58)	-----MSFQRIWAGIT AGAGH FLDRSYQVFKEKLRNPE-LSVNTYVSRAAEVLRMYGL 54
METH2675 (40,61)	-----MSFQRIWAGIT AGAGH FLDRSYQVFKEIKLRNPE-VSVNTFVSRAAGEVLRMYGL 54
MTH1379 (28,45)	-----MRIAWAF AGAGH LLLESVEALEAMVEG--G-HEVTILLSGAAEEVLRMYGL 48
MJ0208 (30,47)	-MNYRFGINMKIIVWCIT AGAGH LLRESFQVMKRLKEEIED-LKVTTLSVRAGEEVVKMYGL 59
MK1484 (31,49)	-----MGLRIGWGIT AGAAH LLVETFKIMKEISRE----HRISAFLLSEAGEEVVRMFGL 49
AF1518 (100,100)	MFEMEKKKKRVWAGIT AGSGD RLPETVDVMLEVKKEFPD-VEIAVYLSKAGAQVVKYKYL 59
	: * : ** : . . : : : : : * : * : * : * : *
MA0696	QKLVKISGGDYLEEIFRESEQSSSPKVGFRFLDRYDALFVTP ATSNT VSKEIAYGIADSL 114
MM1854	QKLVKISGGDYLEEIFRESEQSSSPKVGFRFLDRYDALFVTP ATSNT VSKEIAYGIADSL 114
METH2675	QKLVKISGGDYLEEIFWESEQSSSPKVGFRFLDRYDALFVTP ATSNT VSKEIAYGIADSL 114
MTH1379	ERVKRLSGG--YYRELITDQEGYSFPITGRLSMGRYDLLVSP VTSTNT VAKVVGHIADTL 108
MJ0208	GELYNISNGNYEELILEREHPYSSPITGRLSLGYDYLLIC SPATGNT VAKVVGHIADSL 118
MK1484	DDLREICPGGFYREVFTQSEEGASCPITGRFALGKYDLLIV SPATANT VAKIAHGIADTL 109
AF1518	DTLKENFGA-----VRVEIDANTPFLAGQIQVGRYEFLLI APATSNT VAKIAMGIADSL 113
	: : : * : : : * : . : * : * : * : * : *
MA0696	VTNAVAQAVKGVVYVVPVDIE-GSIISEMPYINIDRK QCRHCET CPPREN CPHGAITEK 173
MM1854	VTNAVSQAVKGRVPVFPVDIE-GSIISEMPYINIDRK QCKHCET CPPREN CPHEAISEK 173
METH2675	VTNAVAQAVKGRVPVYIVPDIE-GSIISEMPYINIDRK QCRHCED CPPREN CPHGAITEK 173
MTH1379	VTNAVAQAGKGVVYVCPVDLEEGDVETVLP SKLELELCRGCEPCLAAAC PEDAIVPG 168
MJ0208	VTNAIAQAGKGVFKSLIVPDYKAGIVTTKLPYAIIDKKK CKLCLKCN ---VCPNGAIVKR 177
MK1484	VTNAVAQAGKGDVPVWVPCDYEEGKIRTITPYMVLRE RCGCGICVD ---ACPRSAIDMV 167
AF1518	LANAAIMGLKAYVPLYIMPSDYKEGEVITKLP----- 145
	: : * : . . * : : * : * : * : : *
MA0696	NGVTDQIELLK CKGCGICKELCPYNAIKGGP -VEVLVRDVMNRNVEIVKGLQGITVLESP 232
MM1854	NGVTDQIDLL CKGCGICKELCPYNAIKGGP -VEVLVRDVMNRNVEIVKSLQGITVLESP 232
METH2675	NGFTDQIDLL CKGCGICKELCPYKAIKGGP -VEVLVRDVIDRNVIVKGLQGITVLESP 232
MTH1379	----VEIRLL SCRCGCACRTAC PHGAVSGGRIITIHMRVDIRNTARLAAMDGIRVFAKP 224
MJ0208	DNF-VEILL SKLGCNCCKKVCYNAIIEGKEIKMRVKIDAENTRKLMELEDVIVLKHP 236
MK1484	DG-KAFIRLL RCVCGCKCAEAC PEDAIHGGLEYEMRVSVDENVRKLERIEGIEVLRHP 226
AF1518	-----DGRDLRLVRKEDVEHVRKLAQMDGVTTILEKP 177
	* : * : * : . . : : : : *
MA0696	EAILELF----- 239
MM1854	EAILELF----- 239
METH2675	EKILDF----- 239
MTH1379	GEILENMAHSHI----- 236
MJ0208	YEILEFFNIR----- 246
MK1484	EEIPERLGELAGEGTPDR 245
AF1518	EDIPKVFVRKHFGKS----- 191

FIG. 1—Continued.

Other ORFs clustered with *afpA* in *M. jannaschii* are annotated as homologs of genes encoding proteins involved in the oxidative stress response of other microbes (Fig. 2). Rubredoxin, encoded by *rd*, is the direct electron donor to rubredoxin-oxygen oxidoreductase in *D. gigas* (7). The *ahp* gene in *E. coli* encodes alkyl hydroperoxide reductase, which is the primary scavenger of endogenous hydrogen peroxide, reducing it to water (24). Rubrerythrin from *Desulfovibrio vulgaris*, encoded by *rr*, also reduces hydrogen peroxide to water (18). Homologs of *isf* were found adjacent to *afpA* homologs in the genomes of *Methanosarcina acetivorans* and *Methanosarcina mazei* with arrangements and orientations identical to those in *A. fulgidus* and *M. jannaschii* (Fig. 2). Furthermore, the *isf* and *afpA* homologs in the *Methanosarcina* species clustered with at least one ORF annotated as the putative homolog of a gene encoding an enzyme that functions in the oxidative stress response of other anaerobes. ORFs clustered with *afpA* in both *Methanosarcina* species are annotated as *dfx* which encodes desulfoferrodoxin from *Desulfovibrio* species that is a two-iron superoxide reductase producing hydrogen peroxide and is proposed to function in the oxidative stress response of anaerobes (2) (15).

Although not clustered with the *afpA* homolog (MTH0697) of *M. thermoautotrophicus* (Fig. 1A), the genome is annotated

with homologs of *isf* (MTH1670 and MTH0122) *fprA* (MTH1350, MTH0220, and MTH0157) and *dfx* (MTH0795). The *M. mazei* genome is also annotated with three *fprA* homologs (MM0891, MM0893, and MM4466), although remote from the *afpA* homolog (MM0635). Finally, the genomes of *A. fulgidus* and *M. jannaschii* are annotated with *dfx* (AF0833 and MJ0741) although they are not clustered with *afpA* homologs (AF1518 and MJ0730) identified in the respective species. Thus, all the sequenced genomes identified with *afpA* homologs also contain ORFs annotated as *dfx* and *fprA*.

Heterologous production, purification, and characterization of AfpA. None of the putative gene products in Fig. 1A or Fig. 1B have been purified and characterized. AfpA produced in *E. coli* was purified to apparent homogeneity as determined by SDS-PAGE (Fig. 3). SDS-PAGE indicated a subunit molecular mass of approximately 23 kDa, in close agreement with the value (21.6 kDa) predicted by the deduced sequence. N-terminal sequencing of the first five residues confirmed that the protein was encoded by AF1518. Native gel filtration chromatography yielded a single peak corresponding to 48 kDa, which approximated the value (43.12 kDa) obtained for the native protein by mass spectrometry and indicates that AfpA is a homodimer (data not shown).

The UV-visible spectrum of the purified protein showed

FIG. 2. Localization of AfpA homologs in finished genomes. Gene annotations and the putative encoded proteins are: *fprA*, flavoprotein A; *isf*, iron-sulfur flavoprotein; *afpA*, archaeoflavoprotein A (this work); *rd*, rubredoxin; *ahp*, alkyl hydroperoxide reductase; *rr*, rubrerythrin; *dfx*, desulfoferredoxin. Horizontal distances between arrows are proportional to the relative spacing of the genes. Open arrows are ORFs annotated as encoding hypothetical proteins. Arrowheads show putative transcription directions. Data were obtained from <http://www.tigr.org>.

absorbance maxima at 272, 379, and 457 with a pronounced shoulder at 480 nm, indicative of a flavin cofactor (Fig. 4). Exposure to air for 1 h had no effect on the spectrum (data not shown), indicating that the flavin cofactor was completely oxidized in the purified protein. After trichloroacetic acid extraction, the flavin was identified as FMN by HPLC analysis (data not shown). The FMN content was found to be 0.8 mol per mol of dimer before reconstitution with FMN and 1.7 mol per mol of dimer after reconstitution, suggesting two noncovalently bound FMN per dimer. Addition of dithionite decreased the absorbance at wavelengths between 300 to 525 nm (Fig. 4) that was completely reversed on exposure to air (not shown). These results show that AfpA is an FMN-containing redox protein. No absorbance was detected at wavelengths above 525 nm during reduction and reoxidation, suggesting that the flavin semiquinone is not stabilized by the protein environment. The molar absorption coefficients for the FMN-reconstituted homodimer at 457 and 375 nm were determined to be 20.8 and 17.3 $\text{mM}^{-1} \text{cm}^{-1}$, respectively. The BLAST search results sug-

gest that AfpA is the prototype of a previously unrecognized family in the domain *Archaea* for which we propose the name archaeoflavoprotein based on the biochemical analyses.

Neither NADH nor NADPH was a direct electron donor for AfpA. Ferredoxin-NADPH oxidoreductase coupled the oxidation of NADPH and reduction of AfpA which was strictly dependent on the presence of ferredoxin (Fig. 5), indicating that ferredoxin is a direct electron donor to AfpA. Rubredoxin was unable to substitute for ferredoxin. Cell extract from lactate-grown *A. fulgidus* catalyzed the CO-dependent reduction of AfpA (Table 1), and the rate was enhanced by the addition of up to 4 μM ferredoxin (Fig. 6). These results establish that AfpA functions as an electron carrier with ferredoxin as the physiological electron donor.

Several compounds were tested as electron acceptors for AfpA by the addition to reaction mixtures containing 18 μM of reduced AfpA and spectrophotometrically monitoring either the oxidation of reduced AfpA or reduction of electron accep-

FIG. 3. SDS-PAGE of purified AfpA. Lane 1, molecular size markers. Lane 2, 10 μg of purified AfpA. The gel was stained with Coomassie R-250.

FIG. 4. UV-visible spectra of AfpA. Spectrum A, as-purified sample (16 μM in 50 mM HEPES, pH 7.2) under 1.0 atmosphere of N_2 . Spectrum B, same as spectrum A except for titration of the absorbance with sodium dithionite.

FIG. 5. Effect of ferredoxin or rubredoxin on the rate of NADP-dependent reduction of AfpA catalyzed by ferredoxin-NADPH oxidoreductase. Symbols: ▲, ferredoxin; ■, rubredoxin.

FIG. 6. Effect of ferredoxin on the rate of CO-dependent reduction of AfpA catalyzed by *A. fulgidus* cell extract.

tor (data not shown). Reduced AfpA was not oxidized by F_{420} (50 μM). Reduced AfpA was fully oxidized by the following electron acceptors (5 and 20 μM) in less than 5 s, which precluded an estimation of rates: 2,3-dimethyl-1,4-naphthoquinone; 2,3-dimethoxy-5-methyl-1,4-benzoquinone; 2-methyl-1,4-naphthoquinone (menadione); 2-hydroxyphenazine; and $\text{K}_3\text{Fe}(\text{CN})_6$. The addition of air-saturated buffer (260 μM O_2 , final concentration) fully oxidized 9 μM reduced AfpA in less than 5 s, also precluding an estimation of the rate. Reduced AfpA was oxidized by ferric citrate (500 μM) at a rate of 22.2 ± 5.2 μM per min. Cytochrome *c* (40 μM) and 2,6-dichlorophenolindophenol (40 μM) were reduced at rates of 87 ± 11 μM and 191 ± 47 μM per min, respectively. However, the simultaneous addition of 20 μM cytochrome *c* and 260 μM O_2 to 9 μM reduced AfpA produced a total of 18.5 ± 1.2 μM reduced cytochrome *c* within 5 s, after which no additional reduction was observed. The accelerated rate of cytochrome *c* reduction in the presence of O_2 precluded a rate determination; nonetheless, the results indicate a rate of at least 222 μM cytochrome *c* per min, which was more than twofold greater than in the absence of O_2 .

These results suggest that superoxide is at least one of the products of O_2 reduction by reduced AfpA and that the superoxide rapidly reduced cytochrome *c* as described for several flavoproteins (19). The same experiment, except with 20 units of superoxide dismutase added per ml, produced 9.5 μM reduced cytochrome *c* within 5 s, after which no further reduction was observed. This result is consistent with the depletion of superoxide available for reduction of cytochrome *c* catalyzed by the dismutase, which further supports that superoxide is a product of the interaction of O_2 with reduced AfpA.

TABLE 1. CO dehydrogenase activity and CO-dependent reduction of AfpA catalyzed by cell extracts

Cell extract	CO-dependent reduction of AfpA ^a (nmol/min/mg)	CO dehydrogenase activity ^b ($\mu\text{mol}/\text{min}/\text{mg}$)
<i>A. fulgidus</i>	20.8–0.78	0.4–0.1
<i>M. thermophila</i> (acetate grown)	22–1.4	1.6–0.12
<i>M. thermophila</i> (methanol grown)	ND	ND
<i>M. thermophila</i> (trimethylamine grown)	ND	ND

^a ND, not detected with a detection limit of 0.01 nmol/min/mg.

^b ND, not detected with a detection limit of 0.01 $\mu\text{mol}/\text{min}/\text{mg}$.

DISCUSSION

The results presented here establish that AF1518 encodes an FMN-containing electron carrier protein (AfpA) with a distinctive FMN-binding motif. The AF1518 ORF was originally annotated as encoding a putative Dfp enzyme. Dfp catalyzes the synthesis of the coenzyme A precursor 4'-phosphopantothenate and is a member of the HFCD superfamily (14). Although sequence analyses indicates that AfpA is related to the HFCD superfamily, the absence of essential motifs indicates that AfpA is not Dfp. Furthermore, the length of AfpA is approximately one-half that of Dfp enzymes, and AfpA is a homodimer, whereas Dfp enzymes are homotrimers (14). Although the cofactor content and size of AfpA are consistent with the properties of a flavodoxin, AfpA clearly is not a flavodoxin. Flavodoxin was not included in the 100 sequences returned by the BLAST search, and AfpA has an FMN binding motif distinct from that of the flavodoxins. Furthermore, flavodoxins are usually monomeric, whereas AfpA is a homodimer.

AfpA is also not a member of the A-type flavoprotein family. The A-type flavoprotein family, with members in both the *Archaea* and *Bacteria* domains, consists of protein sequences (385 to 597 residues) that are greater than twice the length of AfpA and contain signature flavodoxin FMN-binding motifs (31). Moreover, none of the sequences returned by the BLAST search contained members of the A-type flavoprotein family. These results indicate that AfpA is the prototype of a previously unrecognized family of FMN-containing flavoproteins for which the name archaeoflavoprotein is proposed.

The database search also returned a group of deduced sequences with significant identity to AfpA, including the distinctive FMN-binding motif, except for inserts of 52 to 56 residues containing Cys motifs with the potential to ligate two 4Fe-4S clusters (5). Although biochemical characterization is necessary to draw conclusions, these results are at least consistent with a previously unrecognized family of iron-sulfur flavoproteins related to the archaeoflavoprotein family.

The AF1518 ORF was annotated as an enzyme in the pathway of coenzyme A biosynthesis; however, the results presented here clearly establish a physiological role for the gene product (AfpA) as an electron carrier. Moreover, the results establish a physiological role for AfpA in an electron transport chain in *A. fulgidus* for which electrons originating from the CODH/ACS complex are transferred to AfpA mediated by ferredoxin. This role is supported by the results showing that ferredoxin is the direct electron donor to AfpA, and cell ex-

tracts of lactate-grown *A. fulgidus* catalyze the CO-dependent reduction of AfpA that is stimulated by the addition of ferredoxin. During growth of *A. fulgidus* with lactate, the CODH/ACS complex oxidizes the carbonyl group of acetyl-coenzyme A, reducing ferredoxin (10), and also oxidizes CO, which substitutes for the carbonyl group.

The results presented here further suggest that the electron transport chain demonstrated for *A. fulgidus* is also operable in acetate-grown *Methanosarcina* species. Extracts of acetate-grown *M. thermophila* which contain the CODH/ACS complex (30) were shown to catalyze the CO-dependent reduction of AfpA. Furthermore, it is reported (29) that the CODH/ACS complex from *M. thermophila* oxidizes CO and reduces ferredoxin. Finally, the CODH/ACS complex is downregulated during growth of *M. thermophila* on methanol and trimethylamine (30); thus, the inability of extracts from cells grown on these substrates to catalyze the CO-dependent reduction of AfpA is consistent with a role for the CODH/ACS complex in the proposed electron transport chain.

Electrons from the oxidation of lactate by *A. fulgidus* are transferred to a membrane-bound electron transport chain with sulfate as the terminal electron acceptor, generating a proton gradient which drives ATP synthesis. The inventory electron carriers involved are incompletely understood; thus, it would appear that AfpA could have an electron transfer role in energy generation. However, *afpA* homologs were identified in *M. thermoautotrophicus* and *M. jannaschii*, for which the CODH/ACS complex is not a component of energy-yielding pathways (11). Furthermore, although cytochrome *c* and quinones were found to be electron acceptors for AfpA, these electron carriers are not present in *M. thermoautotrophicus* and *M. jannaschii*; thus, the physiological significance of these electron acceptors for AfpA is questionable.

Remarkably, homologs of *afpA* were found only in anaerobic species from the domain *Archaea*, consistent with an electron transport role in a more general process common to these anaerobic species. Indeed, *afpA* in the genome of *A. fulgidus* and the genomes of metabolically diverse methanogenic *Archaea* are clustered with ORFs annotated as encoding enzymes and proteins known to be involved in the oxidative stress response of anaerobes from the domain *Bacteria*. One potential function for AfpA is the reduction of O₂ to less reactive products, which appears to be the preferred approach to the oxidative stress response in anaerobes (2, 15); however, the results presented here suggest that superoxide is a product of the interaction of AfpA with O₂ which argues against a role in reduction of O₂. Nonetheless, the annotated oxidative stress genes *dfx* and *fprA* clustered with *afpA* are putative reductases; thus, a more plausible function for AfpA is that during oxidative stress of *A. fulgidus*, electrons derived from oxidation of the carboxyl group of acetyl-coenzyme A by CODH/ACS are diverted from ferredoxin to AfpA, which participates in electron transport to the reductases.

The clustering of *afpA* with ORFs annotated as *dfx* or *fprA* in methanogenic species implies the same role for AfpA in supplying electrons to the putative reductases in these species. Although the ferredoxin-dependent CODH/ACS complex is not a component in the pathways for conversion of methanol or trimethylamine to methane in *Methanosarcina* species, the final step in the oxidative branch of the pathways generates

reduced ferredoxin (20), affording a potential role for AfpA to participate in supplying electrons to the putative reductases during growth on these substrates. Reduced ferredoxin is also generated by the Ech hydrogenase complex in the first step of the CO₂ reduction pathway for methanogenesis in *M. thermoautotrophicus* and *M. jannaschii* (20), supporting a potential role for AfpA in electron transport to the putative reductases of these species.

Thus, all of the species for which *afpA* homologs have been identified also have mechanisms for generating reduced ferredoxin, consistent with the proposed function for AfpA in coupling the transfer of electrons from ferredoxin to reductases involved in the oxidative stress response. Furthermore, the genomes of all the species in which *afpA* homologs were identified also have annotations for both *dfx* and *fprA*, which is also consistent with an electron transport role for AfpA in the oxidative stress response. Finally, the finding that *isf* homologs are directly adjacent to *afpA* in *A. fulgidus* and several methanogenic species implies electron transport roles for both *Isf* and AfpA.

The results presented here suggest that AfpA is confined to a subset of anaerobes in the domain *Archaea* which is unexplained; however, one consideration is that the putative reductases, AfpA, and other components of an oxidative stress response metabolon coevolved with a high degree of specificity in the last common ancestor to the methanogenic *Archaea* and *Archaeoglobus* species, which have a close phylogenetic and metabolic relationship with the methanogenic *Archaea* (21). Indeed, the genomes of species of anaerobic *Archaea* in which *afpA* homologs were not found (*M. kandleri*, *P. furiosus*, "*P. abyssi*," *P. horikoshi*, and *P. aerophilum*) are also without annotations for *dfx* or *fprA*, the only exception being "*P. abyssi*."

Conclusions. The incorrect annotation of AF1518 in *A. fulgidus* has been rectified and it has been shown that the ORF encodes an electron carrier protein (AfpA) with a distinctive FMN-binding motif. A physiological role for AfpA in *A. fulgidus* has been established by showing that it is linked to the CODH/ACS complex by ferredoxin. Furthermore, genomic analyses imply a broader physiological role for AfpA in responding to oxidative stress. Finally, the results show that AfpA is the prototype of a novel family of flavoproteins, for which the name archaeoflavoprotein is proposed, that appears to be confined to *A. fulgidus* and methane-producing species from the domain *Archaea*.

ACKNOWLEDGMENTS

We thank Frank Cruz for assistance in determination of FMN content and Andrea Gorrell for assistance in generation of the figures.

This work was supported by DOE grant DE-FG02-95ER20198 and NASA-Ames Cooperative Agreement NCC2-1057 to the Pennsylvania State University Astrobiology Research Center.

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