Adenylylsulphate reductase from the sulphate-reducing archaeon *Archaeoglobus fulgidus*: cloning and characterization of the genes and comparison of the enzyme with other iron–sulphur flavoproteins

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Adenylylsulphate (adenosine-5'-phosphosulphate, APS) reductase from the extremely thermophilic sulphate-reducing archaeon *Archaeoglobus fulgidus* is an iron–sulphur flavoprotein containing one non-covalently bound flavin group, eight non-haem iron and six labile sulphide atoms per molecule. Re-evaluation of the enzyme structure revealed the presence of two different subunits with molecular masses of 80 and 18.5 kDa. The subunits are arranged in an $\alpha_2\beta$ subunit structure. We have cloned and sequenced a 2.7 kb segment of DNA containing the genes for the α and β subunits, which we designate *aprA* and *aprB*, respectively. The two genes are separated by 17 bp and localized in the order *aprBA*. While a putative promoter could not be identified in the vicinity of *aprBA* a probable termination signal was found just downstream of the translation stop codon of *aprA*. The codon usage for *aprBA* shows strong preferences for G and C in the third codon position. *aprA* encodes a 73.3 kDa polypeptide, which shows significant overall similarities with the flavoprotein subunits of the succinate dehydrogenases from *Escherichia coli* and *Bacillus subtilis* and the corresponding flavoprotein of *E. coli* fumarate reductase. Part of the homologous peptide stretches could be assigned to domains that are involved in the binding of the substrate or of the FAD prosthetic group. *aprB* encodes a 17.1 kDa polypeptide representing an iron–sulphur protein, seven cysteine residues of which are arranged in two clusters typical of ligands of the iron–sulphur centres in {[Fe₄S₄][Fe₄S₄]} 7-Fe ferredoxins.

Keywords: *Archaeoglobus fulgidus*, adenylylsulphate reductase, dissimilatory sulphate reduction, iron–sulphur protein, flavoprotein

INTRODUCTION

During the past decade the genes for several proteins such as DNA-dependent RNA polymerase, elongation factor 2 and glyceraldehyde-3-phosphate dehydrogenase have been used to point out the phylogenetic relationships

between the three primary domains, the Archaea, the Bacteria and the Eucarya (Zillig *et al.*, 1989; Schröder & Klink, 1991). Because the utilization of sulphur compounds for energy-yielding processes seems to have developed very early during prokaryotic evolution (Trüper, 1982; Schidlowski, 1986) we intend to expand such analyses to bacterial and archaeal sulphur-metabolizing organisms and initiated this work with dissimilatory sulphate reducers (Dahl *et al.*, 1993). While the ubiquitous presence of bacterial desulfuricants in nature has long been observed (Beijerinck, 1895) the discovery of the first archaeal species of this physiological group took until 1987 when K. O. Stetter and co-workers described the extremely thermophilic *Archaeoglobus ful-*

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Abbreviations: APS, adenosine-5'-phosphosulphate; FRD, fumarate reductase; SDH, succinate dehydrogenase.

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gidus (Achenbach-Richter *et al.*, 1987; Stetter, 1988). The organism was isolated from a submarine volcanic hot spring and has an optimum growth temperature of 83 °C.

A. fulgidus has been shown to contain the complete pathway for dissimilatory sulphate reduction known from eubacteria, which requires the sequential action of the enzymes ATP sulphurylase, APS reductase and sulphite reductase (Speich & Trüper, 1988; Dahl *et al.*, 1990). As a key enzyme in dissimilatory sulphur metabolism APS reductase has not only been found in all dissimilatory sulphate-reducing prokaryotes but also in several chemolithoautotrophic and photolithoautotrophic sulphur-oxidizing bacteria of different phylogenetic positions (Trüper, 1989; Takakuwa, 1992). In common with most other APS reductases studied so far the enzyme from *A. fulgidus* has a molecular mass of 160 000 Da and contains FAD, non-haem iron and acid-labile sulphide (Speich & Trüper, 1988). The similarity of bacterial and archaeal APS reductases indicates a strong conservation of structure and function in this protein, making it an ideal candidate for phylogenetic studies.

In this report we mainly intend to supply basic information about this widespread enzyme. We re-evaluate the structure of APS reductase from *A. fulgidus* and describe the cloning and sequencing of the genes encoding the enzyme. The first sequence of an APS reductase should, however, not only contribute to a better understanding of dissimilatory sulphur metabolism but provide the basis for future work on the evolution of this enzyme.

METHODS

Bacterial strains and vectors. *A. fulgidus* DSM 4304^T was grown and harvested as described earlier (Stetter *et al.*, 1987). *E. coli* XL1-Blue, which is *endA1 supE44 hsdR17 (r⁻ m⁺) thi λ⁻ recA1 gyrA96 lac⁻ [F' *proAB lacI^q ΔM15 Tn10 (tet^r)*]*, was the host for the lambda ZAPII (Stratagene) and pBluescript plasmid derivatives and *E. coli* strain P2PLK17 [*supE lac mcrA mcrB hsdR gal* (P2 lysogen)] was the host for lambda FixII (Stratagene) derivatives.

E. coli XL1-Blue was grown in LB medium (Sambrook *et al.*, 1989) with 12.5 µg tetracycline ml⁻¹, which was supplemented with 100 µg ampicillin ml⁻¹ for plasmid containing derivatives. Lambda phage derivatives were plated in top agar consisting of NZCYM (Sambrook *et al.*, 1989) containing 0.7% agarose. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl β-D-thiogalactopyranoside were included in solid media to identify recombinant phage and plasmids containing inserts in the α portion of *lacZ*.

Purification of APS reductase. APS reductase was assayed in the direction of APS formation from sulphite and AMP with ferricyanide as electron acceptor (Speich & Trüper, 1988). Enzyme activity was monitored at 85 °C. Crude extracts were prepared as described previously (Speich & Trüper, 1988). Ammonium sulphate was added to the ultracentrifugation supernatant to 50% saturation at 0 °C. The precipitated protein was separated from the supernatant by ultracentrifugation (140 000 g, 1 h) and discarded. The supernatant was applied to a column of Phenylsepharose CL-4B FastFlow (low substituted) (2.6 × 17 cm; flow rate 3 ml min⁻¹) equilibrated with 50 mM Tris/HCl, pH 8.0, containing ammonium sulphate at

50% saturation. After washing the column with 40% ammonium sulphate in the same buffer the enzyme was eluted with a linear gradient of ammonium sulphate between 40% and 0% saturation (800 ml). APS reductase eluted between 16% and 9% (NH₄)₂SO₄. The active enzyme fractions were pooled and desalted by dialysis against 50 mM Tris/HCl, pH 8.0, followed by chromatography on Q-Sepharose FastFlow (1.6 × 10 cm; flow rate 2 ml min⁻¹) equilibrated in 50 mM Tris/HCl, pH 8.0. With a gradient from 0–400 mM NaCl (600 ml) APS reductase was recovered at 240–280 mM NaCl. Fractions containing the enzyme were combined, desalted by dialysis and further purified by chromatography on MonoQ HR 5/5. The column was equilibrated with 50 mM Tris/HCl, pH 8.0, and after application of the protein washed with the same buffer containing 100 mM NaCl. Via a linear gradient from 100 mM to 300 mM NaCl (20 ml) APS reductase was retrieved at 190–230 mM NaCl with a purity coefficient of 5.0 (*A*₂₈₀/*A*₃₉₀) and a specific activity of 2.3 µmol APS min⁻¹ mg⁻¹.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed according to the procedure of Laemmli (1970) and bands were stained with Coomassie Blue.

Protein sequencing. Purified native APS reductase of *A. fulgidus* was directly sequenced by automated Edman degradation in a Beckman sequencer. For amino-terminal sequencing of the α subunit the contents of a 12.5% SDS-polyacrylamide gel loaded with 50 µg APS reductase were electrophoretically transferred onto a siliconized glass filter and directly subjected to automated Edman degradation with an Applied Biosystems 477A gas-phase sequencer.

Recombinant DNA techniques. All general cloning techniques were performed according to standard methods (Sambrook *et al.*, 1989). Chromosomal DNA of *A. fulgidus* was obtained by lysis with 0.5% SDS as described elsewhere (Dahl *et al.*, 1993). The mixture was extracted with phenol and chloroform and the DNA was purified by CsCl density gradient centrifugation. Exonuclease III deletion clones were prepared using the nested deletion kit from Pharmacia and sequenced with the chain-termination method (Sanger *et al.*, 1977) using T7 Polymerase and [α-³²P]dATP or *Taq* polymerase and dye-labelled dideoxynucleotides. Dye-labelled DNA was analysed with a 373A DNA Sequencer from Applied Biosystems. The DNA sequence data were aligned and analysed with the DNASIS software from Pharmacia.

Construction of DNA libraries. A complete library of chromosomal DNA of *A. fulgidus* was constructed as described by Dahl *et al.* (1993). A second *A. fulgidus* library was constructed from size-fractionated *Hind*III fragments of 1–1.5 kb, which were filled-in with dATP and dGTP using the DNA polymerase Klenow fragment and ligated into lambda ZAPII, which had been digested with *Spe*I and filled-in with dCTP and dTTP. This library was screened directly without amplification. pBluescript derivatives were prepared from lambda ZAPII by using the R408 single-stranded helper phage according to the protocol provided by Stratagene.

Hybridizations. DNA probes for Southern blot experiments and screening of phage libraries were radioactively labelled by the random-priming method (Feinberg & Vogelstein, 1983) with [α-³²P]dCTP. Hybridizations were performed overnight at 68 °C in 5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 8.0), 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.1% SDS, containing 100 µg nicked and denatured calf thymus DNA ml⁻¹. Membranes and filters were washed twice for 15 min each at room temperature in 2 × SSC, 0.1% SDS, and then three times for 15 min each at 68 °C in 0.1 × SSC, 0.1% SDS.

Oligonucleotide probes were radioactively labelled by T4 polynucleotide kinase with [γ - 32 P]ATP. Hybridizations were carried out at 42 °C overnight in the solution described above. The membranes were washed four times for 15 min each at 42 °C in 5 $\times$ SSC, 0.1 % SDS.

Calculations. The amino acid sequences were analysed on the Convex at the DKFZ Heidelberg using the FASTA program (Devereux *et al.*, 1984) and the Swiss-Prot Protein Data Base (EMBL, Heidelberg).

RESULTS AND DISCUSSION

Purification of APS reductase and re-evaluation of the enzyme structure

APS reductase from *A. fulgidus* has been described as an iron-sulphur flavoprotein consisting of two identical 80 kDa subunits (Speich & Trüper, 1988). We developed an improved purification scheme that takes advantage of the high resolution of FPLC, thereby eliminating the need for preparative isoelectric focusing. The purified enzyme exhibited a specific activity of 2.3 U mg⁻¹ compared to the 2.39 U mg⁻¹ obtained with the original purification procedure. When analysed by SDS-PAGE the APS reductase preparations were found to contain a Coomassie-stainable band with a mobility corresponding to a molecular mass of ~ 18.5 kDa in addition to the 80 kDa polypeptide (Fig. 1). Subunits of comparable size have also been described for the APS reductases from *Desulfovibrio vulgaris*, *Desulfovibrio gigas* and *Thermodesulfobacterium mobilis* (Bramlett & Peck, 1975; Fauque *et al.*, 1986; Kremer *et al.*, 1988; Lampreia *et al.*, 1990). By integration of densitometric scans and correction for molecular mass a stoichiometry

Fig. 1. SDS-PAGE of purified *A. fulgidus* APS reductase. A 2 μ g sample of APS reductase was applied to the gel. Molecular masses of markers are indicated.

of 2.5:1 was obtained for the 80 kDa (α) to 18.5 kDa (β) subunits. Together with the apparent molecular mass of the holoenzyme, i.e. 160 000, an $\alpha_2\beta_2$ structure is suggested for the native archaeal APS reductase. The small subunit is lost during isoelectric focusing and is not essential for enzyme activity with ferricyanide as an artificial electron acceptor since APS formation (the enzyme is assayed in the unphysiological direction of APS formation) was proven for enzyme preparations lacking the 18.5 kDa peptide (Speich & Trüper, 1988). The peptide might, however, be necessary for reaction of APS reductase with its *in vivo* electron donor. Cloning and sequencing (see below) revealed that the two genes encoding the two different subunits of *A. fulgidus* APS reductase are separated by only 17 bp and are probably localized in the same operon, indicating that the small subunit is likely to be an integral part of the enzyme.

Cloning and nucleotide sequence analysis of the genes for APS reductase

Amino-terminal sequencing of native APS reductase gave two different sequences of 24 and 5 residues. These two sequences could unambiguously be distinguished on the basis of their relative yields which were close to 2:1. Peptide sequence analysis of the α subunit alone gave an amino acid sequence identical to the major sequence obtained for the native enzyme.

A mixture of 128 different 20-mer oligonucleotides, which was designed from residues 3 to 9 (YYPKKYE) of the amino-terminus of the α subunit, was used to analyse Southern blots of *A. fulgidus* DNA. A single hybridizing band at 1.3 kb was detected for *Hind*III-digested DNA. Therefore a non-amplified λ ZAPII library of 1–1.5 kb *Hind*III fragments was screened with the same probe. One of the 1500 recombinant clones from the library gave a positive signal and was found to contain a 1.3 kb insert which was subsequently cloned into pBluescript I SK. This clone was designated pARS1. A 697 bp length of the 3'-terminus of the pARS1 insert was sequenced on both strands and found to carry an open reading frame (ORF) of 453 bp encoding a 150 residue, 17.1 kDa peptide. Amino acids 2–6 of this peptide match the minor sequence obtained by Edman degradation of native APS reductase, indicating that the corresponding ORF is the gene for the β subunit, which we designate *aprB*. Downstream (17 bp) of *aprB*, 138 bp of the 5'-terminus of a second ORF were detected encoding the complete chemically determined amino-terminal amino acid sequence of the α subunit of APS reductase. These findings indicated that this truncated ORF was part of the gene for the α subunit, which we designate *aprA*. In order to clone the complete gene the amplified λ FixII library of *A. fulgidus* (Dahl *et al.*, 1993) was screened with the radioactively labelled 1.3 kb *Hind*III fragment as a probe. Of 20 000 recombinant clones from this library, 10 gave positive signals and were found to contain the same 14 kb insert.

The nucleotide sequence of both strands of a 2720 bp portion of the phage insert was determined (Fig. 2). In

(1) ATACCTCCACCAGATATAG

aprB →

20 AGTAATTTAATTTTTTTTAGAAAAATGTATGAAAAGGTTGGAGGTGAGTTGAATGCCATCGTTTGTAAA
(1) *rbs* M P S F V N

90 CCCGAAAAGTGTGACGGATGTAAGGCGTTGGAGAGAACGGCGTGTGAGTACATCTGCCCCAACGACTTG
(7) P E K C D G C K A L E R T A C E Y I C P N D L

160 ATGACACTCGATAAAGAGAAGATGAAAGCGTACAACAGAGACCCGGACATGTGCTGGGAGTGCTACAGCT
(30) M T L D K E K M K A Y N R D P D M C W E C Y S C

230 GTGTTAAGATGTGCCCGCAGGGGCAAATTGACGTGAGAGGCTACGTGGACTACTCCCCGCTTGGCGGTGC
(54) V K M C P Q G Q I D V R G Y V D Y S P L G G A

300 CTGTGTGCCAATGAGAGGAACCTCAGACATCATGTGGACGGACAAGTACAGAAACGGAAAGGTTCTGAGG
(77) C V R M R G T S D I M Y T D K Y R N G K V L R

370 TTCAAGTTCGCCATCAGAACAACCCCGTGGGGCTCAATTCAGCCCTTCGAGGGATTCCCTGAGCCGACCG
(100) F K F A I R T T P W G S I Q P F E G F P E P T E

440 AGGAGGCATTGAAGAGCGAACTGCTTGTCTGGAGAACCCGAGATTATTGGAACGAGCGAGTTCCCGCAGGT
(124) E A L K S E L L A G E P E I I G T S E F P Q V

aprA →

510 AAAGAAGAAGGCTTAAAGGTGAGGTGGTAGAAATGGTATATTATCCGAAAAAGTATGAGTTGTATAAGGC
(1) K K K A END *rbs* M V Y Y P K K Y E L Y K A

580 AGATGAAGTGCCGACAGAGGTTGTGGAGACGGACATCTTGATTATCGGAGGAGGTTTCTCCGGCTGTGGT
(14) D E V P T E V V E T D I L I I G G G F S G C G

HindIII

650 GCAGCGTACGAGGCTGCCTACTGGGCAAAGGTTGGCGGTTTGAAGCTTACGCTTGTGAGAAAGCAGCAG
(37) A A Y E A A Y W A K V G G L K L T L V E K A A V

720 TTGAGAGAAGCGGAGCTGTTGCCAGGGTCTTTTCAGCCATTAACACATACATCGACCTTACCGGCAGGTC
(61) E R S G A V A Q G L S A I N T Y I D L T G R S

790 CGAGAGGCAGAACACCCCTTGAGGATTACGTCAGATACGTCACCCTCGACATGATGGGATTGGCGAGAGAG
(84) E R Q N T L E D Y V R Y V T L D M M G L A R E

860 GACCTTGTGTGCTGACTACGCAAGGCATGTTGACGGAACGGTCCACCTCTTCGAGAAGTGGGGACTGCCCCA
(107) D L V A D Y A R H V D G T V H L F E K W G L P I

930 TCTGGAAGACTCCCGATGGGAAGTACGTCAAGAGAGGGACAGTGGCAGATAATGATTCACGGGTGAGAGC
(131) W K T P D G K Y V K R G T V A D N D S R V R A

1000 TACAAGCCAATCATCGCAGGAAGCTGGAAGGATGGAAGTCGGTAAGGAGGAACATCTACGAGAGAGTTTT
(154) T S Q S S Q E A G R M E V G K E E H L R E S F

1070 TCATCTTCCGAGCTTCTGAAGGGCAAGAACGACCCCAACGCTGTGGCCGAGCCGTCGGTTTCAGCGTTA
(177) S S S E L L K G K N D P N A V A G A V G F S V R

1140 GAGAGCCCCAAGTTCTACGTGTTCAAGGCGAAAGCCGTCATTCTGGCAACCGGAGGTGCAACACTGCTCTT
(201) E P K F Y V F K A K A V I L A T G G A T L L F

1210 CAGGCCGAGAAGCACTGGCGAAGCAGCAGGAAGGACATGGTATGCAATCTTCGACACTGGCAGCGGTTAC
(224) R P R S T G E A A G R T W Y A I F D T G S G Y

1280 TACATGGGCTTGAAGGCCGAGCGATGCTCACGCAGTTTGAACACCGCTTCATACCCCTCAGGTTCAAGG
(247) Y M G L K A G A M L T Q F E H R F I P F R F K D

1350 ACGGTTACGGCCAGTTGGAGCATGGTTCCTGTTCTTCAAGTGTAAGGCCAAGAACGCGTATGGAGAGGA
(271) G Y G P V G A W F L F F K C K A K N A Y G E E

1420 GTACATCAAGACAAGGGCTGCAGAGCTTGAGAAGTACAAGCCCTACGGTGCAGCCAGCCAATCCCGACA
(294) Y I K T R A A E L E K Y K P Y G A A Q P I P T

Fig. 2. For legend see facing page.

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1490   CCGCTGAGAAACCACCAGGTCATGCTCGAAATCATGGACGGCAACCAGCCAATCTACATGCACACTGAGA
(317)   P L R N H Q V M L E I M D G N Q P I Y M H T E R
1560   GGCTTCTCGCTGAGCTGGCTGGAGGAGACAAGAAGAAGCTGAAGCACATCTACGAGGAGGCTTTTCGAGGA
(341)   L L A E L A G G D K K K L K H I Y E E A F E D
1630   CTTCTCGACATGACAGTCAGCCAGGCTCTGCTGTGGGCTGCCAGAACATCGACCCGAGGAGCAGCCG
(364)   F L D M T V S Q A L L W A C Q N I D P Q E Q P
1700   TCTGAAGCTGCACCGGCTGAGCCCTACATCATGGGTTACACAGCGGTGAGGCAGGTTTCTGGGTATGCG
(387)   S E A A P A E P Y I M G S H S G E A G F W V C G
1770   GTCCTGAGGATCTGATGCCGGAGGAGTACGCAAAGCTCTTCCCGCTGAAGTACAACAGGATGACCACAGT
(411)   P E D L M P E E Y A K L F P L K Y N R M T T V
1840   CAAGGGACTCTTCGCCATCGGTGACTGTGCTGGTGCCAACCCGCACAAGTTCTCCAGGGTTCGTTCACTG
(434)   K G L F A I G D C A G A N P H K F S R V R S L
1910   AGGCAGGATTGTAGCGAAGGCGCAGTGATGTTTCATCCCGAGCAGAAGCCCAACCCAGAAATTGACGATG
(457)   R Q D C S E G A V M F I P E Q K P N P E I D D A
1980   CGGTCGTTGAGGAACCTAAGAAGAAGGCTACGCACCGATGGAGAGGTTTCATGCAGTACAAGGACCTCTC
(481)   V V E E L K K K A Y A P M E R F M Q Y K D L S
2050   AACTGCCGATGACGTCAACCCAGAGTACATCCTGCCGTGGCAGGGTCTTGTCAGGTCGAGAAGATCATG
(504)   T A D D V N P E Y I L P W Q G L V R L Q K I M
2120   GACGAGTATGCTGCTGGAATTGCAACAATCTACAAGACCAACGAGAAGATGCTGCAGAGAGCTCTTGAGC
(527)   D E Y A A G I A T I Y K T N E K M L Q R A L E L
2190   TGCTCGCCTTCCTGAAGGAGGACCTCGAGAAGCTCGCTGCAAGAGACCTCCACGAGCTGATGAGAGCATG
(551)   L A F L K E D L E K L A A R D L H E L M R A W
2260   GGAGCTTGTCACAGAGTCTGGACTGCTGAGGCACACGTCAGGCACATGCTCTTCAGAAAGGAAACCAGA
(574)   E L V H R V W T A E A H V R H M L F R K E T R
2330   TGGCCCGGATACTACTACAGAACCGACTACCCAGAGCTCAACGACGAGGAGTGGAAGTGCTTCGTCGCA
(597)   W P G Y Y Y R T D Y P E L N D E E W K C F V C S
2400   GCAAGTACGACGCTGAGAAGGACGAGTGGACCTTCGAGAAAGTGCCGTACGTGCAGGTCATCGAGTGGAG
(621)   K Y D A E K D E W T F E K V P Y V Q V I E W S
2470   CTTCTAAAGCTCTAAATTTTTTCTTTTTTCTAAATCTAAAAGTTTTTATTGCATTGGCTTAACCTCTGCAT
(644)   F END          tx term
2540   CATGTACTTGGAACCTGCCATTGAGAGGCTAAATGCCAAGGAGCTTGAGGAGAATACAGGAGCGCAAGC
2670   TCAAGGCCTGGGTAAGCACAAAGTCTATCAAGTATTCCTCCCTTCTTCGCCAGAGGGTTCAAGGAGGCTG
2680   GAGTAACAACCTGACGACATCAAAAGTCTGAACGATTGGCCC

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Fig. 2. Nucleotide sequence of the *A. fulgidus apr* locus. Deduced amino acid sequences for two open reading frames, designated *aprB* and *aprA*, are shown below the sequence. Nucleotides are numbered beginning at the first position of the sequence and amino acid positions are shown in parentheses. The following features are indicated: *Hind*III sites; tx term, putative transcription termination signal; rbs, putative ribosome binding sites. The underlined amino acid sequences correspond to the amino-terminal peptide sequences that were chemically determined from purified APS reductase.

addition to *aprB* (residues 73–525) an ORF of 1935 bp (*aprA*) spanning residues 542–2476 was identified which encodes a 644 residue, 73.3 kDa peptide. The lack of an amino-terminal methionine in both mature peptides indicates that it is removed during post-translational processing. The molecular masses deduced for the *aprA* and *aprB* encoded peptides are in good agreement with the values obtained for the α and β subunits by SDS-PAGE analysis. A molecular mass of 163700 Da can be calculated for an $\alpha_2\beta_2$ -structured APS reductase, which is in close agreement with the value obtained by gel filtration (Speich & Trüper, 1988).

The 54.2% G + C content of the *aprBA* region is higher than the value of 46% found for *A. fulgidus* total DNA (Stetter *et al.*, 1987). The codon usage of *aprBA*, with preference for G and C in the third base position, is similar to that in the *dsrAB* genes (Dahl *et al.*, 1993).

As shown in Fig. 2 each of the two ATG initiation codons is preceded by a putative ribosome-binding site (RBS), at least 6 nucleotides of which are complementary to 9 nucleotides at the 3'-end of the 16S rRNA of *A. fulgidus* (UCCUCCACU; Achenbach-Richter *et al.*, 1987). The high degree of complementarity of the RBS to the 3'-

Fig. 3. For legend see facing page.

terminus of 16S rRNA may reflect the capacity of the cell to express the APS reductase genes at a high level, since this enzyme accounts for more than 2% of the total cellular soluble protein when the organism is grown on sulphate (Speich & Trüper, 1988). Comparable to *dsrA* and *dsrB* from *A. fulgidus*, the strong preference for C in codons of the type NNC/T observed in *aprA* may also favour efficient translation, as has been proposed for the *mcr* genes of *Methanococcus vannielii* (Cram *et al.*, 1987).

A sequence perfectly representing the 2-fold repetition of a conserved heptanucleotide (TTTTTYT), which has been proposed to be a transcription termination signal in extremely thermophilic sulphur-dependent archaea (Reiter *et al.*, 1988), is located 11 nucleotides downstream of the TAA stop codon of *aprA*. Neither *aprA* nor *aprB* are preceded by sequences showing significant homology to consensus promoters proposed for archaea (Brown *et al.*, 1989), indicating that these two genes are probably part of a larger transcriptional unit. We consider it to be a likely possibility that the APS reductase genes form an operon with those encoding ATP sulphurylase. This enzyme catalyses sulphate activation to adenylylsulphate which then serves as the substrate of APS reductase. The genes encoding dissimilatory sulphite reductase (*dsrA* and *dsrB*), which catalyses the final step of the sulphate reduction pathway, form a separate transcriptional unit (Dahl *et al.*, 1993). This could enable the cell to transcribe *dsrAB* independently from *aprBA* and the genes for ATP sulphurylase and to express these genes differentially under different growth conditions.

Peptides encoded by *aprA* and *aprB*

Unlike terminal oxidases in aerobic respiration, the reductases of the sulphate-reducing prokaryotes including *A. fulgidus* are soluble enzymes that are not tightly associated with the cytoplasmic membrane (LeGall & Fauque, 1988; Speich & Trüper, 1988). Growth of these organisms on lactate and sulphate, however, requires that at least part of the reducing power be transferred from the membrane-localized electron-transport chain to APS reductase, and it has been suggested that menaquinone serves as the electron donor of APS-reductase-catalysed adenylylsulphate reduction (Kunow & Thauer, 1993). A hydropathy analysis profile (Kyte & Doolittle, 1982) of the peptides encoded by *aprA* and *aprB* (not shown) did not reveal markedly hydrophobic segments that would be likely to mediate membrane integration. This does not, however, exclude reaction of the soluble APS reductase

with membrane-bound electron carriers, as has been discussed for dissimilatory sulphite reductase from the same organism (Dahl *et al.*, 1993).

Amino acid sequence data has been obtained for only one other APS reductase. Comparison with the chemically determined amino acid sequence of the α subunit of *Thermodesulfobacterium commune* APS reductase (M. Walbröl, personal communication) showed identities at 7 of 24 amino acids sequenced, indicating that these two peptides are homologous (not shown).

Comparison of the *aprA* encoded peptide to the sequences in the Swiss-Prot Data Base using the program FASTA revealed significant homology with the flavoprotein subunits of several bacterial and eukaryotic FAD-containing reductases and dehydrogenases. Fig. 3 shows an alignment of the amino acid sequence deduced from *aprA* with those of the flavoprotein subunits of fumarate dehydrogenase (FRD) from *E. coli* (Cole, 1982) and the succinate dehydrogenase (SDH) from *Bacillus subtilis* (Phillips *et al.*, 1987) and *E. coli* (Wood *et al.*, 1984). The overall similarity between the archaeal and the bacterial flavopeptides varies between 29 and 30% when matches between similar amino acids are included (after Dayhoff *et al.*, 1978); the percentage of identical amino acids is 15 to 17%.

The alignment of the four peptides reveals that the conserved regions are not statistically distributed but are concentrated in six segments (marked by lines and numbered in Fig. 3). Four of these segments can be assigned to domains that are involved in binding the FAD prosthetic group: Segment 1 at the amino-terminal end of the flavoproteins corresponds to part of a consensus sequence for ADP binding sites derived by Wierenga *et al.* (1986). More specifically, the sequences compared here match a fingerprint proposed to be diagnostic for amino acid sequences that form a $\beta\alpha\beta$ -fold with binding properties for the ADP moiety of FAD or NAD(P). While the length of the consensus sequence is not constant due to length variability of a loop between the α -helix and the second β -strand, 11 positions have been defined for which certain amino acids are required as structure-forming residues. Three glycine residues at positions 6, 8 and 11, and an acidic residue at the end of the fingerprint are especially important and therefore designated as 'core'-fingerprint (Wierenga *et al.*, 1986). As shown in Fig. 4, residues 24–56 of the deduced APS reductase sequence match the fingerprint including the core residues at 10 positions and therefore most probably serve as the binding site for the ADP portion of FAD. In analogy with the

Fig. 3. Alignment of the deduced amino acid sequence of *aprA* from *A. fulgidus* with those of the flavoprotein (Fp) subunits of three bacterial dehydrogenases. Amino acid positions are indicated. The abbreviations are: BsSDH, *Bacillus subtilis* succinate dehydrogenase (Phillips *et al.*, 1987); EcSDH, *E. coli* succinate dehydrogenase (Wood *et al.*, 1984); EcFRD, *E. coli* fumarate dehydrogenase (Cole, 1982); AfAPR, *A. fulgidus aprA*-encoded peptide. The proposed APS binding site is indicated. Arrows show the potential active site histidine and arginine residues in SDH and FRD. Shaded boxes indicate positions at which similar or identical amino acids are present in all four sequences. For this comparison the following are considered similar: aspartate, glutamate, asparagine and glutamine (D, E, N and Q); arginine, histidine and lysine (R, H and K); isoleucine, valine, methionine and leucine (I, V, M and L); phenylalanine, tyrosine and tryptophan (F, Y and W); serine, threonine, proline, alanine and glycine (S, T, P, A and G) (Dayhoff *et al.*, 1978). Segments of particularly high homology are numbered and highlighted by lines.

Secondary structure	β β β β β β	α α α α α α α α α α α α α α α α	L O O P β β β β β β
		* * *	*
Amino acid fingerprint	K A x A x G x G x x x A x x A x x x x x x x x x A x A x D x		
	R I I	I I	I I E
	H L L	L L	L L
	S V V	V V	V V
	T M M	M M	M M
	Q C C	C C	C C
	N		
AfAPR Fp 24–56	D <u>I</u> L <u>I</u> I <u>G</u> <u>G</u> <u>G</u> F S <u>G</u> C G A <u>A</u> Y E <u>A</u> A Y W A K V G G L K <u>L</u> T <u>L</u> V <u>E</u> K		
AfAPR Fp 265–292	F R F K D <u>G</u> Y <u>G</u> P V <u>G</u> A W F <u>L</u> F F K C K A K N - - - - <u>A</u> Y G E <u>E</u> Y		

Fig. 4. Potential ADP binding folds in the deduced amino acid sequence of *aprA*. The sequences were aligned with a fingerprint sequence that folds into a $\beta\alpha\beta$ -structure with ADP binding properties (Wierenga *et al.*, 1986). Amino acids which match the fingerprint are underlined. Positions of the 'core' fingerprint are marked by asterisks.

three-dimensional structure of human glutathione reductase (Guest & Rice, 1984; Rice *et al.*, 1984) the GGG-turn found at position 30–32 of the *aprA* deduced sequence is in close contact with the pyrophosphate group of the FAD.

The ADP-binding domain is immediately followed by another segment (2) of particularly high sequence conservation. In all but the *aprA*-encoded peptide a conserved histidine residue is found in this region. This residue has been shown to be involved in covalent linkage of the flavin portion of the FAD cofactor (Kenney *et al.*, 1972; Cole, 1982; Wood *et al.*, 1984; Phillips *et al.*, 1987). The conserved histidine has been replaced by cysteine, serine, arginine or tyrosine via site-directed mutagenesis in *E. coli* fumarate reductase resulting in an enzyme retaining activity but containing an FAD molecule which is non-covalently bound to the enzyme (Blaut *et al.*, 1989). Equivalent substitutions in D-nicotinic acid oxidase from *Arthrobacter oxidans* also resulted in loss of covalent attachment of the FAD (Mauch *et al.*, 1989). In the peptide deduced from *aprA* the conserved histidine is replaced by a glycine residue, indicating that the FAD prosthetic group is not covalently bound in the archaeal APS reductase. Indeed, previous results clearly showed that the flavin moiety is not covalently linked to the enzyme (Speich & Trüper, 1988).

Another example of a flavoprotein related to fumarate reductases and succinate dehydrogenases but containing non-covalently bound FAD is the soluble flavocytochrome *c* of *Shewanella putrefaciens* (Pealing *et al.*, 1992). Around the flavin attachment site the amino acid sequence of this protein is, however, not related to the enzymes with covalently bound flavin. The archaeal APS reductase, therefore, appears to be the first enzyme with non-covalently bound FAD that lacks the conserved histidine residue but contains a region homologous to the FAD attachment site in fumarate reductases and succinate dehydrogenases. Implications of our finding on the evolution of covalent binding of FAD will have to be discussed in the future when more APS reductase

Amino acid fingerprint	T x x x x I Y I I G D
	M V W V V
	A F A A
	L L
AfAPR Fp 431–441	<u>T</u> T V K G <u>L</u> <u>F</u> <u>A</u> <u>I</u> <u>G</u> <u>D</u>

Fig. 5. Potential binding site of the ribityl chain of FAD in the deduced amino acid sequence of *aprA*. The sequence is aligned with a fingerprint proposed by Eggink *et al.* (1990). Amino acids which match the fingerprint are underlined.

sequences are available for comparative studies. As pointed out by Hederstedt (1987), the primary sequence around the histidine is not conserved in all proteins with histidine-bound FAD. This suggests that the conserved sequence is more important for enzyme function than for binding of the flavin.

The sequence at positions 394 to 453 of the *aprA*-encoded peptide, which comprises two highly conserved segments (4 and 5), is homologous to an amino acid sequence in human glutathione reductase forming part of the FAD binding pocket (Cole *et al.*, 1985). Segment 5 contains a stretch of amino acids matching a fingerprint that has been proposed to be indicative of sites which bind the ribityl chain of the flavin moiety (Eggink *et al.*, 1990). Fig. 5 shows an alignment of the *aprA*-deduced peptide with this fingerprint. Studies on the three-dimensional structure of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (Schreuder *et al.*, 1988), human glutathione reductase (Karplus & Schulz, 1987) and lipoamide dehydrogenase from *Azotobacter vinelandii* (Schierbeek *et al.*, 1989) revealed that the conserved Asp at the end of the fingerprint forms hydrogen bonds with the O-3 group of the ribityl chain of the flavin moiety. It is therefore likely that Asp-441 has the same function in APS reductase.

Quantitative measurements showed the presence of only 1 mol FAD per mol of *A. fulgidus* APS reductase holoenzyme composed of one β and two flavoprotein subunits

Fig. 6. Alignment of the cysteine clusters predicted by *aprB* to those of 7-Fe ferredoxins (Fdx). Sequences of subunits of the following enzymes and ferredoxins are aligned: Av, *Azotobacter vinelandii* (Stout, 1988); Tt, *Thermus thermophilus* (Sato *et al.*, 1981); Sg, *Streptomyces griseus* (Trower *et al.*, 1990); Ba, *Bacillus acidocaldarius* (Schlatter *et al.*, 1985); Af APR, *A. fulgidus* APS reductase. Cysteine ligating clusters I and II, respectively, are indicated (after Iismaa *et al.*, 1991). Shaded boxes indicate positions at which similar or identical amino acids are present in all four sequences. Amino acid similarity groups cf. Fig. 3.

(Speich & Trüper, 1988). Since some flavin loss may have occurred during enzyme preparation we cannot fully exclude that the native archaeal APS reductase carries one FAD prosthetic group per α subunit. It should, however, be taken into consideration that all other APS reductases studied so far have independently been reported to contain either one FAD per enzyme molecule or one FAD per eight iron atoms (cf. the review by Fauque *et al.*, 1991). Exactly the same stoichiometries are observed for the *A. fulgidus* enzyme. We therefore want to point out the possibility that the FAD binding site present in each of the two α subunits might represent a form of 'half of sites reactivity' comparable to the flavoprotein of assimilatory sulphite reductase from *E. coli* where binding sites for FAD as well as for FMN are proposed to function in a similar manner (Ostrowski *et al.*, 1989). In addition to the binding sites of the FAD cofactor there are two more highly conserved segments (3 and 6) with as yet unknown function.

APS reductases from dissimilatory sulphate reducers have been reported to be inhibited by sulphhydryl reagents (Peck *et al.*, 1965; Stille & Trüper, 1984), indicating that thiol groups may be essential for catalysis. None of the eight cysteine residues in the *aprA*-deduced sequence is strictly conserved in all four flavoproteins compared in Fig. 2 and only one of them (Cys-442) occupies a comparable position in *E. coli* FRD and *B. subtilis* SDH. This residue is, however, not conserved in most other succinate dehydrogenases and fumarate reductases (Pealing *et al.*, 1992) and identification of potentially essential cysteines will have to await sequencing of further APS reductases. The only strictly conserved histidine residue (His-448 in the *aprA*-deduced sequence) has been suggested to be catalytically important and may perform a proton donor-acceptor function during catalysis. This residue is probably located close to the isoalloxazine ring of the FAD prosthetic group (Vik & Hafeti, 1981; Hederstedt & Hafeti, 1986; Hederstedt, 1987).

In the region between residues 255 and 330 APS reductase shows little homology to bacterial SDH and FRD. An active site histidine and a substrate-binding arginine residue (H-232 and R-248 of *E. coli* fumarate reductase) are localized in the corresponding regions of SDH and FRD, indicating that this portion of the polypeptides is

responsible for substrate binding and specificity (Schröder *et al.*, 1991). With respect to the different metabolic functions of the enzymes in comparison one has to expect a different sequence in the corresponding region of APS reductase. Indeed, we found a second region resembling an ADP binding site in this part of the sequence. This stretch of amino acids (residues 265–292) perfectly fits the 'core' fingerprint proposed by Wierenga *et al.* (1986) and matches the whole fingerprint at six of eleven positions (Fig. 4). The three-dimensional structure of this region may therefore resemble the typical ADP-binding $\beta\alpha\beta$ -fold. Since the substrate of APS reductase, adenosine-5'-phosphosulphate, is structurally very similar to ADP this part of the enzyme could represent at least part of the substrate binding site. Other proteins with two nucleotide binding sites are oxidoreductases that besides FAD bind NAD or NADP. Comparable to the second site in those proteins the APS binding site proposed by us is located approximately in the middle of the *aprA*-encoded sequence.

Previous results have demonstrated the presence of eight non-haem iron and six acid-labile sulphur atoms within *A. fulgidus* APS reductase (Speich & Trüper, 1988). The peptide encoded by *aprB* contains nine cysteine residues, eight of which occur in two clusters. The spacing of these cysteine residues resembles that of 7-Fe ferredoxins (Fig. 6), including the ferredoxin from *Azotobacter vinelandii* which is known from X-ray studies to contain an $[\text{Fe}_3\text{S}_4]$ and an $[\text{Fe}_4\text{S}_4]$ centre (Stout, 1988). Similarities between this ferredoxin and *A. fulgidus* APS reductase extend to electrochemical properties: both contain one iron-sulphur cluster easily reducible by dithionite (Cluster I) and an $[\text{Fe}_4\text{S}_4]$ centre (Cluster II) which only partially reacts with this reductant at higher pH (Lampreia *et al.*, 1991; Iismaa *et al.*, 1991). It is interesting to note in this regard that oxidized APS reductase shows electron paramagnetic resonance (EPR) spectra of a minor paramagnetic species reminiscent of $[\text{Fe}_3\text{S}_4]$ clusters (Lampreia *et al.*, 1991). However, the main species of clusters in the reduced state in *A. fulgidus* APS reductase show EPR spectra clearly identifying them as $[\text{Fe}_4\text{S}_4]$ centres. Interconversion of $[\text{Fe}_3\text{S}_4]$ to $[\text{Fe}_4\text{S}_4]$ clusters in proteins purified aerobically as was the case for APS reductase have repeatedly been described and the question whether

[Fe₃S₄] clusters are due to oxidative damage or indeed do have biological meaning has not been settled (Bruschi & Guerlesquin, 1988; George *et al.*, 1989). Analogous to *A. vinelandii* ferredoxin, cysteines 13, 21 and 51 of the *aprB*-encoded peptide probably ligate cluster I which corresponds to the [Fe₃S₄] centre in the *A. vinelandii* protein, whereas Cys-25, Cys-47, Cys-50 and Cys-53 bind cluster II. If APS reductase contains two [Fe₄S₄] clusters *in vivo* each of these requires four ligands. The *aprB*-encoded peptide contains an additional cysteine at position 10 which could serve as a fourth thiolate ligand for a [Fe₄S₄]-structured cluster I. It has, however, been proposed that the corresponding residue in *Thermus thermophilus* ferredoxin is extraneous and not involved in binding of an iron-sulphur cluster (George *et al.*, 1985). Alternatively, cluster I of the *aprB*-encoded peptide could include a non-cysteine ligand in its coordination, possibly water or hydroxyl from solvent as has been shown for activated pig heart aconitase (Robbins & Stout, 1989).

A notable difference between the sequence deduced from *aprB* and the *Bacillus acidocaldarius* ferredoxin sequence and those of the other 7-Fe ferredoxins shown in Fig. 6 is the absence of an otherwise strictly conserved cysteine residue (Cys-24 in the *A. vinelandii* sequence). In the *A. vinelandii* protein this residue is in Van der Waals contact with cluster II (Stout, 1988). By site directed mutagenesis it was shown, however, that it is neither essential for assembly of a functional [Fe₄S₄]-structured cluster II nor required for liganding cluster I (Iismaa *et al.*, 1991).

Concluding remarks

The genes encoding the two different subunits of APS reductase from the extremely thermophilic archaeon *A. fulgidus* were isolated and the DNA sequence determined. This appears to be the first reported characterization of APS reductase genes. The sequence data obtained classified the archaeal APS reductase as a typical iron-sulphur flavoprotein with significant homology to its bacterial counterparts and allowed identification of probable substrate and cofactor binding sites. In the future, comparative amino acid sequence analyses should not only provide estimates of the evolutionary divergence of APS reductases and information about the evolution of sulphur metabolism in general but also about the evolution of FAD and iron-sulphur cluster binding sites.

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