Dissimilatory sulphite reductase from *Archaeoglobus fulgidus*: physico-chemical properties of the enzyme and cloning, sequencing and analysis of the reductase genes

CHRISTIANE DAHL,¹* NICHOLAS M. KREDICH,² RAINER DEUTZMANN³ and HANS G. TRÜPER¹

¹Institut für Mikrobiologie und Biotechnologie, Rheinische Friedrich-Wilhelms-Universität Bonn, 53115 Bonn 1, Germany

²Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA

³Institut für Biochemie, Universität Regensburg, 93053 Regensburg, Germany

(Received 1 March 1993; revised 26 April 1993; accepted 28 April 1993)

A dissimilatory sulphite reductase was isolated from the extremely thermophilic dissimilatory sulphate-reducing archaeon *Archaeoglobus fulgidus*. In common with other dissimilatory sulphite reductases thus far characterized, the enzyme has an $\alpha_2\beta_2$ -structure and contains sirohaem, non-haem iron atoms and acid labile sulphide. The oxidized enzyme exhibited absorption maxima at 281, 394, 545 and 593 nm with a weak band around 715 nm. We have cloned and sequenced the genes for the α and β subunits of this enzyme, which we designate *dsrA* and *dsrB*, respectively. They are contiguous in the order *dsrA dsrB* and probably comprise an operon, since *dsrA* is preceded by sequences characteristic of promoters in methanogenic archaea, and *dsrB* is followed by a sequence resembling termination signals in extremely thermophilic sulphur-dependent archaea. *dsrA* and *dsrB* encode 47.4 kDa and 41.7 kDa peptides, which have 25.6% amino acid sequence identity, indicating that they may have arisen by duplication of an ancestral gene. Each deduced peptide contains cysteine clusters resembling those postulated to bind sirohaem- $[\text{Fe}_4\text{S}_4]$ complexes in sulphite reductases and nitrite reductases from other species. The *dsrB* encoded peptide lacks a single cysteine residue in one of the two clusters, suggesting that only the α subunit binds a sirohaem- $[\text{Fe}_4\text{S}_4]$ complex, and chemical analyses showed the presence of only two sirohaems per $\alpha_2\beta_2$ enzyme molecule. Both deduced peptides also contain an arrangement of cysteine residues characteristic of $[\text{Fe}_4\text{S}_4]$ ferredoxins, and chemical analyses were consistent with the presence of six $[\text{Fe}_4\text{S}_4]$ clusters per $\alpha_2\beta_2$ enzyme molecule, two of which would be expected to be associated with sirohaem while the other four could bind to the ferredoxin-like sites.

Introduction

The dissimilatory sulphate-reducing prokaryotes are a large and extremely diverse physiological group of anaerobic organisms, which includes bacteria and the archaeal species *Archaeoglobus fulgidus* (Achenbach-Richter *et al.*, 1987; Stetter, 1988), and *A. profundus* (Burggraf *et al.*, 1990). All dissimilatory sulphate-reducers investigated thus far contain the enzyme sulphite reductase, which catalyses the terminal step in

sulphate reduction, the reduction of sulphite to sulphide. Dissimilatory type sulphite reductases have been found to consist of two different polypeptides in an $\alpha_2\beta_2$ structure and to contain sirohaem, non-haem iron and acid-labile sulphide (Peck & Lissolo, 1988; LeGall & Fauque, 1988; Fauque *et al.*, 1990). Recently a third type of subunit, termed γ , has been identified in the *Desulfovibrio vulgaris* dissimilatory sulphite reductase, for which a subunit structure of $\alpha_2\beta_2\gamma_2$ has been proposed (Pierik *et al.*, 1992). All these enzymes are probably closely related to the assimilatory sulphite reductases of *Escherichia coli* and *Salmonella typhimurium* (Murphy *et al.*, 1973; Murphy & Siegel, 1973); the 'low-spin' sulphite reductases of *D. vulgaris* (Huynh *et al.*, 1984), *Desulfuromonas acetoxidans* and *Methanosarcina barkeri* (Moura *et al.*, 1986); the dissimilatory-type 'reverse'

* Author for correspondence. Tel. 228 735591; fax 228 737576.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M95624.

sulphite reductases of the thiobacilli and the phototrophic sulphur bacterium *Chromatium vinosum* (Schedel *et al.*, 1975, 1979); and the ammonia-producing sirohaem nitrite reductases of higher plants (Hucklesby *et al.*, 1976; Vega & Kamin, 1977), algae (Zumft, 1972), fungi (Vega *et al.*, 1975) and *E. coli* (Jackson *et al.*, 1981). In common with the dissimilatory sulphite reductases these enzymes all contain sirohaem at their catalytic centres.

Dissimilatory sulphite reductases from a great variety of species have been studied extensively and four types of this enzyme are differentiated on the basis of their spectral and redox properties: desulfoviridin (Lee & Peck, 1971), desulforubidin (Lee *et al.*, 1973), P₅₈₂ (Trudinger, 1970) and desulfofusicidin (Hatchikian & Zeikus, 1983). These studies have, however, been confined to enzymes from sulphate reducers belonging to the bacterial domain. We intend to expand these analyses to dissimilatory sulphite reductases of archaeal origin and have initiated this work with *A. fulgidus*, one of the two extremely thermophilic archaeal species of the sulphate-reducers. The organism belongs to the kingdom of the Euryarchaeota (Woese *et al.*, 1990) and is grouped with the Methanomicrobiales/extreme halophile cluster (Woese *et al.*, 1991). *A. fulgidus* was isolated from a volcanic hot spring and has an optimum growth temperature of 83 °C (Achenbach-Richter *et al.*, 1987; Stetter *et al.*, 1988). The organism was shown to carry out dissimilatory sulphate reduction via the pathway originally proposed for bacterial species (Speich & Trüper, 1988; Dahl *et al.*, 1990), which requires the sequential action of the enzymes ATP sulphurylase, APS reductase and sulphite reductase (Peck, 1962).

In this report we present the purification of dissimilatory sulphite reductase from *A. fulgidus* and examine a number of its properties. Furthermore, we describe the cloning and sequencing of the two genes that encode the α and β subunits of this enzyme. By comparing their deduced amino acid sequences with those of other sirohaem-binding proteins, we identified putative binding sites for prosthetic centres in the archaeal sulphite reductase. We further attempted to rationalize the implications of homologies between sirohaem-binding proteins for future understanding of the evolution of this class of enzymes.

Methods

Bacterial strains, plasmids and media. *A. fulgidus* [VC16, DSM 4304 (Stetter, 1988)] was grown as described by Stetter *et al.* (1987) and obtained from K. O. Stetter (Universität Regensburg, Germany) as frozen packed cells. *E. coli* NM522 [*supE thi Dhsd5 Δ(lac-pro)(F' pro⁺ lacIqZ ΔM15)*] and *E. coli* P2PLK17 [*supE lac mcrA mcrB hsdR gal* (P2 lysogen)] were hosts for pT7T3 phagemid and lambda FixII (Stratagene) derivatives, respectively. *E. coli* XL1-Blue, which is *endA1 supE44 hsdR17 (r⁻ m⁺) thi λ⁻ recA1 gyrA96 lac⁻ [F' proAB lacI^q ZDM15*

Tn10 (*tet^r*), was the host for the lambda ZAPII (Stratagene) and pBluescript plasmid derivatives.

E. coli XL1-Blue was grown in LB medium (Sambrook *et al.*, 1989) supplemented with 12.5 µg tetracycline ml⁻¹, and NM522 was grown on double strength YT medium (Miller, 1972), which was supplemented with 100 µg ampicillin ml⁻¹ for plasmid containing derivatives and 70 µg kanamycin ml⁻¹ for the production of single-stranded phagemid DNA (Mead *et al.*, 1986). 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 (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were included in solid media to identify recombinant phage and plasmids containing inserts in the α portion of *lacZ*.

Assays and spectroscopic measurements. Sulphite reductase activity in crude extracts was assayed in cuvettes sealed with rubber stoppers at 85 °C with N₂ as a gas phase. Additions were made with microlitre syringes. In a volume of 0.9 ml the assay contained extract in 50 mM-potassium phosphate buffer, pH 7.0, 0.5 mM-dithioerythritol (DTE), 50 mM-potassium phosphate buffer, pH 7.0, and 2 mM electrochemically reduced methylviologen. The reactions were started by addition of 100 µl of oxygen-free sulphite solution (100 mM-Na₂SO₃ in 50 mM-potassium phosphate buffer, pH 7.0, 0.5 mM-EDTA). The disappearance of methylviologen was monitored by following the decrease in absorbance at 600 nm ($\epsilon = 13.0 \text{ cm}^2 \mu\text{mol}^{-1}$). During purification procedures sulphite reductase was followed by measuring the absorbances at 394, 545 and 593 nm and the enrichment was monitored by the A_{280}/A_{593} ratio. Concentrations of sulphite reductase are based on a molecular mass of 178 200 Da, which was calculated from the deduced amino acid sequence of the α and β subunits and the $\alpha_2\beta_2$ structure of the enzyme.

Protein determinations were done according to the method of Lowry using bovine serum albumin as a standard. The non-haem iron content of sulphite reductase was determined by the method of Massey (1957). Acid-labile sulphide was determined according to King & Morris (1966). Sirohaem content was measured by the method of Siegel *et al.* (1978).

Purification of sulphite reductase. Unless otherwise noted all purification steps were performed aerobically at room temperature in 50 mM-Tris/HCl, pH 8.0, containing 1 mM-β-mercaptoethanol and 0.5 mM-EDTA. Crude extracts were prepared as described earlier (Speich & Trüper, 1988). The supernatant of a first ultracentrifugation was desalted on Sephadex G-25 and ammonium sulphate was added to 40% saturation at 0 °C. The precipitated protein was separated from the supernatant by ultracentrifugation (140 000 g, 4 °C). The supernatant was applied to a Pharmacia-FPLC system and portions of 1000 mg total protein were separated on a Phenylsepharose CL-4B FastFlow column (1.5 × 18 cm) equilibrated with 40% ammonium sulphate in standard buffer. Using a linear decreasing gradient from 40% to 0% ammonium sulphate (370 ml) the sulphite reductase eluted between 21% and 14% ammonium sulphate. The combined fractions containing the enzyme had a purity index of 16 (A_{280}/A_{593}) and were dialysed against standard buffer. For further purification, portions of less than 20 mg total protein were passed through a MonoQ HR 5/5 column equilibrated with standard buffer. The column was washed with standard buffer containing 280 mM-NaCl. Via a linear gradient from 280 mM- to 380 mM-NaCl (20 ml) the sulphite reductase was recovered at 310–330 mM-NaCl with a purity coefficient of 6.2. The fractions containing the enzyme were pooled and desalted. The ion exchange chromatography was repeated resulting in an electrophoretically homogeneous protein (A_{280}/A_{593} 5.4; A_{280}/A_{394} 2.18). Approximately 40 mg of pure sulphite reductase were obtained from 40 g of cells.

The molecular mass of the native enzyme was estimated by gel filtration on Superose 6 equilibrated with standard buffer containing

150 mM-NaCl. The proteins of the Boehringer Combithek II (no. 104558) were used as references. The isoelectric point of sulphite reductase was determined by analytical isoelectric focusing on Servalyt Precotes 3-6 using protein mixture 9 from Serva for calibration of the pH gradient.

Protein sequencing. Subunits of highly purified *A. fulgidus* sulphite reductase were separated by PAGE in SDS. For N-terminal amino acid sequencing, subunits were electrophoretically transferred onto siliconized glass-fibre membranes (Glassybond, Biometra), visualized by Coomassie Brilliant Blue staining, excised and subjected to automated Edman degradation with an Applied Biosystems 477A gas-phase sequencer. For tryptic digests, bands were excised from the gel, washed with 90% ethanol, lyophilized, soaked in 0.2 M-NH₄HCO₃ and digested with 1 mg trypsin per 100 µl overnight at 37 °C. Tryptic peptides were extracted from the gel matrix with additional 0.2 M-NH₄HCO₃, and this solution was lyophilized. The residue was dissolved in 6 M-guanidine.HCl, and peptides were fractionated on a Vydac C₁₈ reversed phase HPLC column and sequenced.

Antibody production and immunoassays. A 0.6 mg portion of purified *A. fulgidus* sulphite reductase in Freund's incomplete adjuvant was injected intramuscularly into the neck of a young, immunologically naive, female New Zealand White rabbit. Booster injections of 0.15 mg of sulphite reductase without adjuvant were administered at 34 and 42 d, and serum was obtained 6 d later. Specific antibodies to both sulphite reductase subunits were demonstrated by immunoblot analysis (Sambrook *et al.*, 1989) with the purified enzyme and with crude extracts of *A. fulgidus*. Contaminating cross-reactivity could not be detected.

Recombinant DNA methods. General methods were those described by Sambrook *et al.* (1989). Portions of cloned DNA were subcloned into the polylinker region of pT7T3 and sequenced by the dideoxy-chain termination method (Sanger *et al.*, 1977) from either single-stranded or double-stranded DNA templates. Oligodeoxynucleotides were prepared with a model 380A automated DNA synthesizer from Applied Biosystems. The polymerase chain reaction (PCR) was performed with a reagent kit from Perkin Elmer Cetus. Reaction mixtures contained 5 ng of template DNA, 100 pmol of each oligodeoxynucleotide primer, and 2.5 U of Taq polymerase in 100 µl 10 mM-Tris/HCl (pH 8.3), 50 mM-KCl, 1.5 mM-MgCl₂, 0.2 mM of each deoxynucleotide triphosphate, 0.01% gelatine and were incubated for 25 cycles at 93 °C for 0.5 min, 55 °C for 1.0 min and 72 °C for 1.5 min. After a final cycle at 93 °C for 0.5 min, 55 °C for 1 min and 72 °C for 3 min, mixtures were extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and precipitated with ethanol.

To obtain unsheared genomic DNA of *A. fulgidus*, 1 g frozen cell paste was suspended in 5 ml of 10 mM-Tris/HCl (pH 8.0), 10 mM-Na₂EDTA. Proteinase K at 20 µg ml⁻¹ and 0.5% SDS were added, and cells were lysed by incubation at room temperature for 120 min with occasional swirling. The mixture was then extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and chloroform/isoamyl alcohol (24:1, v/v), and DNA was purified by CsCl density gradient ultracentrifugation.

Construction of DNA libraries. *A. fulgidus* DNA was partially digested with *Sau*III and restriction sites were partially filled in with dGTP, dATP and the Klenow fragment of *E. coli* DNA polymerase. After size fractionation on an agarose gel, 9 to 20 kb fragments were ligated into lambda FixII, which had been linearized with *Xho*I and partially filled in with dCTP and dTTP. Ligated DNA was packaged *in vitro* with a phage lambda packaging extract (Gigapack Gold, Stratagene), and phage were plated on *E. coli* P2PLK17 at a density of approximately 2×10^3 p.f.u. per 150 cm² Petri dish. The insert frequency was 70%. Phage were collected by washing the plates, and this amplified library was re-plated for screening.

A second *A. fulgidus* library was constructed from *Hind*III fragments, which were filled in with Klenow fragment and blunt-end ligated to an *Not*I/*Eco*RI adaptor. Size-fractionated fragments of 3 to 4 kb were ligated into lambda ZAPII, which had been digested with *Eco*RI and dephosphorylated. After *in vitro* packaging the library was plated on *E. coli* XL1-Blue at a density of 1×10^3 p.f.u. per 150 cm² Petri dish. Approximately 5×10^5 plaques were obtained with an insert frequency of 90%. This library was screened directly without amplification.

Screening of DNA libraries. Libraries were plated at 1 to 10×10^3 p.f.u. per 150 cm² Petri dish, and after 8 to 16 h at 37 °C plaques were transferred and bound to nylon membranes by UV irradiation. For screening with DNA probes, filters were prehybridized for 5 h at 42 °C in 5 × SSC (1 × SSC is 0.15 M-NaCl, 15 mM-sodium citrate, pH 8.0), 1 × Denhardt's solution, 0.1% SDS, containing 200 µg ml⁻¹ of nicked and denatured salmon sperm DNA (Sargent, 1987) and 50% formamide. Hybridization was performed for 12-16 h at 42 °C in the same solution lacking the salmon sperm DNA but containing 0.5 mg sodium pyrophosphate ml⁻¹ and random-primed ³²P-labelled DNA probe (Feinberg & Vogelstein, 1983) at 5×10^6 c.p.m. ml⁻¹. Filters were washed three times for 30 min each at 23 °C in 2 × NET (1 × NET is 0.15 M-NaCl, 1 mM-EDTA, 15 mM-Tris/HCl, pH 8.3), 0.1% SDS, and then twice for 45 min at 60 °C in 0.2 × NET, 0.1% SDS.

For screening with an oligonucleotide probe, filters were prehybridized for 5 h at 38 °C in 6 × NET, 6 × Denhardt's solution, 0.1% SDS and 200 µg ml⁻¹ of nicked and denatured salmon sperm DNA. Hybridization was carried out for 16-20 h at 38 °C in the same solution lacking salmon sperm DNA but containing 0.5 mg sodium pyrophosphate ml⁻¹ and 0.02 µg ³²P-labelled oligodeoxynucleotide ml⁻¹ (5×10^8 c.p.m. µg⁻¹). Filters were washed three or four times for 30 min each at 23 °C in 6 × NET, 0.1% SDS and then once for 7 min at 40 °C in 2 × NET, 0.1% SDS.

Positive plaques were identified by radioautography and purified by re-plating. Lambda phage DNA was prepared by the method of Chisholm (1989), and inserts were subcloned in pT7T3 (Pharmacia-LKB) for restriction mapping and DNA sequencing. pBluescript derivatives were prepared from lambda ZAPII vectors according to the protocol provided by Stratagene.

Sequence comparisons. Protein sequences were compared to those in GenBank by the programs BestFit, Compare, DotPlot and Gap from the Sequence Analysis Software package of the Genetics Computer Group, University of Wisconsin (Devereux *et al.*, 1984). Multiple sequence alignments were based on the BestFit program and finally refined by visual inspection to select those alignments that retained the maximum sequence continuity.

Results

Purification and physicochemical characterization of sulphite reductase

Cell extracts of *A. fulgidus* were found to catalyse sulphite-dependent oxidation of reduced methylviologen with a specific activity of 70 mU (mg protein)⁻¹ at 85 °C. This activity decreased to 10% when the reaction temperature was lowered to 65 °C and no activity was found below 55 °C. Thus, the sulphite reducing enzyme of *A. fulgidus* appeared to be extremely thermophilic, comparable to ATP sulphurylase and APS reductase from the same organism (Speich & Trüper, 1988; Dahl *et al.*, 1990). The sulphite reducing activity coincided with

Fig. 1. Absorption spectra of oxidized (—) and reduced (····) *A. fulgidus* sulphite reductase. The spectra of a 3.8 μ M-sulphite reductase solution in 50 mM-Tris/HCl, pH 8.0, were recorded under nitrogen at room temperature.

the presence of a green protein with absorption spectra similar to those obtained for dissimilatory sirohaem-sulphite reductases (LeGall & Fauque, 1988; Fauque *et al.*, 1990). The enzyme was purified to homogeneity and shown to constitute 0.5% of the total cellular soluble protein. The high concentration of the protein in crude extracts supports a dissimilatory function. The relative molecular mass of the enzyme was found to be 218 000 Da by gel filtration. On SDS gel electrophoresis it presented two bands of 40 and 50 kDa. A small 11 kDa subunit has recently been found in desulfoviridin from *D. vulgaris* (Pierik *et al.*, 1992). SDS-PAGE analyses of *A. fulgidus* sulphite reductase on high-percentage gels designed to detect a polypeptide of comparably small size clearly showed that the archaeal enzyme does not contain a third subunit. Our results indicate that the native protein is a tetramer with a quaternary structure of $\alpha_2\beta_2$. The isoelectric point of sulphite reductase was determined to be 4.2 by analytical isoelectric focusing.

The oxidized enzyme exhibited absorption maxima at 281, 394, 545 and 593 nm with shoulders at 430 and 625 nm (Fig. 1). Upon reduction with dithionite, the α -band was shifted to 598 nm while its absorption decreased and the Soret peak shifted from 394 to 390 nm. Furthermore, the spectrum of oxidized sulphite reductase showed a weak band around 715 nm which disappeared after reduction. This band indicates that the sirohaem in the archaeal enzyme is mainly in the high-spin state (Stolzenberg *et al.*, 1981), a feature shared by all dissimilatory sulphite reductases investigated (LeGall & Fauque, 1988; Fauque *et al.*, 1990). The presence of high-spin ferric haem in the protein is supported by

preliminary electron paramagnetic resonance spectroscopy. The molar extinction coefficients of *A. fulgidus* sulphite reductase at 281, 394 and 593 nm were estimated to be 395 000, 184 000 and 60 000.

Upon extraction with acetone hydrochloride (Siegel *et al.*, 1978) the haem prosthetic group of the enzyme exhibited absorption peaks at 592.5 and 366 nm, similar to sirohaem preparations obtained from other sulphite reductases (Siegel *et al.*, 1978; Fauque *et al.*, 1990). After transfer to pyridine the extracted chromophore yielded a spectrum comparable to that of the *E. coli* sulphite reductase sirohaem (Siegel *et al.*, 1978). Quantification of the data indicated the presence of 2 mol sirohaem per mol of the archaeal sulphite reductase. This result is confirmed by the extinction coefficient at the Soret peak which is close to half that of desulfofusicidin from *Thermodesulfobacterium commune*. This enzyme has been reported to contain four instead of two sirohaem groups per molecule (Hatchikian & Zeikus, 1983). An analysis for acid labile sulphide gave a value of 20 mol sulphide per mol sulphite reductase. The complete release of iron from the enzyme required precipitation of the protein with trichloroacetic acid prior to analyses (Massey, 1957). The determination resulted in a value of 22–24 non-haem Fe atoms per enzyme molecule. The physico-chemical properties of *A. fulgidus* sulphite reductase are summarized in Table 1 and compared to those of the thermophilic enzyme from *T. commune* (desulfofusicidin) and the mesophilic sulphite reductases from *D. baculatus* (desulforubidin) and *D. gigas* (desulfoviridin).

Cloning of the genes for sulphite reductase

Peptide sequence analyses of the α subunit of sulphite reductase gave an amino-terminal sequence of 35 residues and sequences for six tryptic peptides for a total of 115 residues. The amino-terminal sequence could not be obtained from the β subunit, suggesting that it is blocked, but two tryptic peptides were sequenced for a total of 28 residues. A mixture of 32 different 20-mer oligonucleotides, which was designed from the amino acid sequence FMFFEKD of one of the tryptic peptides of the α subunit, was used to screen the amplified lambda FixII library of *A. fulgidus* DNA. Each of ten positive clones isolated from this library contained the same 18.4 kb insert, which was found to consist of a 470 bp portion of *A. fulgidus* DNA encoding the FMFFEKD peptide and an 18 kb fragment of lambda DNA that represented a cloning artefact. This clone was designated PLD1. Our failure to isolate more than a single clone from this library prompted us to construct a second library. Southern blot analyses of *A. fulgidus* DNA showed reactivity of a 3.2 kb *Hind*III fragment with the probe to the FMFFEKD sequence. Therefore, a lambda

Table 1. Comparison of *A. fulgidus* sulphite reductase with desulforubidin, desulfofusicidin and desulfovirodin

References: 1, Hatchikian & Zeikus (1983); 2, Lee *et al.* (1973); 3, Moura *et al.* (1988); 4, Fauque *et al.* (1991); 5, Lee & Peck (1971); 6, Peck & LeGall (1982).

Property	<i>Archaeoglobus fulgidus</i>	<i>Thermodesulfobacterium commune</i> ¹ Desulfofusicidin	<i>Desulfovibrio baculatus</i> ^{2,3,4} Desulforubidin	<i>Desulfovibrio gigas</i> ^{3,5,6} Desulfovirodin
Absorption maxima (nm); molar extinction coefficients ($\times 10^3$; $\text{cm}^2 \mu\text{mol}^{-1}$)	394; 184 593; 60	389; 310 576; 89	392; NG 545; NG	390; NG 408; 150 580; NG 628; 50
Mol. mass (Da)	178 200	167 000	225 000	200 000
Subunit mol. mass (Da)	2 α (47 400) 2 β (41 700)	2 α (47 000) 2 β (47 000)	2 α (NG) 2 β (NG)	2 α (42 000)† 2 β (50 000)†
Subunit composition	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$
Non-haem iron*	22–24	20–21	21 $\pm$ 2‡	18 $\pm$ 2‡
Acid-labile sulphide*	20	16	14.7	14
Sirohaem*	2	4	2	2§

NG, Not given.

* Atoms or groups per $\alpha_2\beta_2$ -structured enzyme molecule.

† For *Desulfovibrio vulgaris*.

‡ Total iron.

§ Sirohydrochlorin.

ZAPII library of 3 to 4 kb *Hind*III fragments was screened with a probe consisting of a PCR-generated 364 bp *Taq*I fragment from PLD1, which contained only *A. fulgidus* sequence. Ten positive clones were isolated and found to contain the same 3.17 kb insert.

Nucleotide sequence analysis

The cloned *Hind*III fragment was completely sequenced on both strands and was found to contain 3175 bp with two open reading frames of 1257 bp and 1101 bp (Fig. 2). The upstream ORF-1 encodes a 418 residue, 47.4 kDa peptide which contains sequences identical to all those chemically determined for the *A. fulgidus* sulphite reductase α subunit. These findings indicate that ORF-1 is the gene for the α subunit, which we designate *dsrA*. The lack of an amino-terminal methionine in the mature peptide indicates that it is removed during post-translational processing. ORF-2 is downstream of and contiguous with *dsrA* and contains an ATG initiation codon that overlaps the *dsrA* termination codon in the sequence TAATG. ORF-2 encodes a 366 residue, 41.7 kDa peptide containing 28 amino acids corresponding to those found in two chemically sequenced tryptic peptides of the β subunit. We conclude that ORF-2 is the gene for the β subunit, which we designate *dsrB*. The region downstream of *dsrB* does not contain open reading frames of more than 60 bp in length. The molecular masses deduced for the *dsrA* and *dsrB* encoded peptides are in good agreement with the values obtained

for the α and β subunits by SDS-PAGE analysis. A molecular mass of 178 200 Da can be calculated for the native $\alpha_2\beta_2$ -structured sulphite reductase, which is close to the value obtained by gel filtration.

The entire DNA fragment has a G+C content of 48.4 mol %, which is very similar to the value of 46 % found for *A. fulgidus* total DNA (Stetter *et al.*, 1987). The codon usage of *dsrAB* is characteristic for *A. fulgidus* (Speich, 1991). Codons AAG (87 %, Lys), AAC (91 %, Asn), AGA/G (97 %, Arg), CAG (100 %, Gln), CAC (94 %, His), TAC (75 %, Tyr) and TTC (84 %, Phe) are used preferentially. As previously observed in other members of the Archaea (Cue *et al.*, 1985; Reeve *et al.*, 1986), the dinucleotide CG is under-represented in coding regions, and codons such as AGA and ATA, which are rare in *E. coli*, occur frequently in these two *A. fulgidus* genes.

As shown in Fig. 2, both the ATG start codons of *dsrA* and *dsrB* are immediately preceded by putative ribosome-binding sites (RBS), at least 7 nucleotides of which are complementary to a sequence of 13 nucleotides at the 3'-end of the 16S rRNA of *A. fulgidus* (UCCUCCACUAGGU) (Achenbach-Richter *et al.*, 1987). The high degree of complementarity of the RBS to the 3' terminus of 16S rRNA may reflect the capacity of the cell to express the sulphite reductase genes at a high level, since enzymes of dissimilatory sulphur metabolism account for up to 7 % of the total cellular soluble protein (Peck & LeGall, 1982). The strong preference in *dsrA* and *dsrB* for C in codons of the type NNC/T may also

A putative promoter was identified in the 84 bp of 5'-untranslated sequence preceding *dsrA*. Starting 56 nucleotides upstream of the translational start codon, the sequence AAATTCTTATATA occurs, which is identical at 12 of 13 positions with the box A motif of the putative consensus promoter in methanogenic archaea (Brown *et*

al., 1989). Twenty one bp downstream of the box A sequence, the heptanucleotide TGCAATT is found, which is identical at 6 of 7 positions to the box B consensus sequence of promoters in methanogenic archaea (Brown *et al.*, 1989). The distance between these two boxes is usually 18 to 24 bp (Hausner *et al.*, 1991), and transcription initiation is thought to occur at the second position of the box B sequence (Wich *et al.*,

Fig. 3. Alignment of the deduced amino acid sequences of *dsrA* and *dsrB* and those in seven other sirohaem-[Fe₄S₄] binding proteins obtained with the aid of the program BestFit (Devereux *et al.*, 1984) and a modified version (Gribskov & Burgess, 1986) of the amino acid weighting matrix of Schwartz & Dayhoff (1978). Amino acid positions are indicated. The abbreviations are: DvSir, *Desulfovibrio vulgaris* low *M_r*, 'low-spin' sulphite reductase (Tan *et al.*, 1991); EcNirB, *E. coli nirB* NADH-linked nitrite reductase (Peakman *et al.*, 1990); AsNir, *Aspergillus nidulans* nitrite reductase (Kinghorn & Campbell, 1989); AnSir, *Anacystis nidulans* (Synecococcus PCC 7942) ferredoxin-linked sulphite reductase (G. Gisselmann & J. D. Schwenn, personal communication); EcSirHp, haemoprotein component of *E. coli* assimilatory sulphite reductase (Ostrowski *et al.*, 1989b); SpiNir, spinach nitrite reductase (Back *et al.*, 1988); asrC, *Salmonella typhimurium* asrC encoded peptide for an anaerobically expressed sulphite reductase (Huang & Barrett, 1991); dsrA and dsrB, *A. fulgidus* *dsrA* and *dsrB* encoded peptides; aa, amino acid residues. Arrows show the highly conserved cysteine residues proposed to coordinate sirohaem-[Fe₄S₄] binding. Shaded boxes indicate positions at which similar or identical amino acids are present in at least five of the nine 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).

1988). The 734 bp 3'-untranslated portion of our sequence contains the heptanucleotide TTTTTT beginning 23 bp downstream of the *dsrB* termination codon. This is the only occurrence of the sequence TTTTTYT, which has been proposed to be a tran-

scription termination signal in the extremely thermophilic, sulphur-dependent archaea (Reiter *et al.*, 1988) and in the extremely thermophilic methanogenic archaeon *Methanothermobacter fervidus* (Weil *et al.*, 1988). The presence and position of putative promoter and ter-

mination sequences indicate that *dsrA* and *dsrB* comprise a single transcription unit and that the two genes are coordinately expressed.

Peptides encoded by *dsrA* and *dsrB*

A dot matrix analysis (Schwartz & Dayhoff, 1979; Devereux *et al.*, 1984; Gribskov & Burgess, 1986) revealed significant homology between the peptides predicted from *dsrA* and *dsrB*. The program Gap (Devereux *et al.*, 1984) gave an alignment with 91 identical residues (25.6%) with 11 gaps and an overall similarity of 50% when matches between similar amino acids were included.

The most noteworthy feature of the predicted *dsrA* and *dsrB* peptides was the presence of two groups of cysteine-containing clusters closely resembling the (Cys-X₅-Cys)-X_n-(Cys-X₃-Cys) arrangement that has been proposed to co-ordinate the binding of sirohaem and a [Fe₄S₄] cluster in the assimilatory sulphite reductases from *E. coli* and *S. typhimurium* (Ostrowski *et al.*, 1989b). This arrangement extends from residue 176 to 224 in the *dsrA* peptide and from residue 134 to 182 in the *dsrB* peptide, where residue 134 is Thr instead of Cys. Such clusters are also found in other sirohaem-dependent reductases, namely *D. vulgaris* 'low-spin' sulphite reductase (Tan *et al.*, 1991), *Anacystis nidulans* (*Synechococcus* PCC 7942) ferredoxin-linked sulphite reductase (G. Gisselmann & J. D. Schwenn, personal communication), and the nitrite reductases of *E. coli* (Peakman *et al.*, 1990), *Aspergillus nidulans* (Kingham & Campbell, 1989) and spinach (Back *et al.*, 1988). Furthermore, the same cysteine-arrangement is present in the *asrC* encoded peptide, which is proposed to be the sirohaem-containing subunit of anaerobically expressed sulphite reductase from *S. typhimurium* (Huang & Barrett, 1991).

An alignment of the proposed sirohaem-[Fe₄S₄] binding regions from nine peptides shows the highest degree of similarity around the Cys-X₃-Cys motif, which is preceded by Gly in all but the *dsrB* encoded peptide (Fig. 3). Less similarity is found in the Cys-X₅-Cys sequences, where the *dsrB* encoded peptide contains a Thr in place of the first Cys residue. In six of the nine sequences, including that of the *dsrA* peptide, the first Cys in the cluster is preceded by Ala. Position X₂ in the Cys-X₅-Cys cluster contains Gly in seven peptides and either Ser or Ala in the other two, suggesting a requirement for a small residue at this position. The other four residues separating the cysteines vary considerably among these peptides and may function only to form a loop that must 'get out of the way' of the sirohaem-[Fe₄S₄] complex (Ostrowski *et al.*, 1989b). All nine sequences contain a conserved acidic amino acid located 6 residues after the first cysteine

cluster and 7 to 8 residues after the second cluster. Additional similarities were found in a region beginning 54 to 70 residues before the Cys-X₅-Cys cluster and ending just past the Cys-X₃-Cys cluster. Similarities between *dsrA*, *dsrB* and *asrC* were found all the way to the carboxy-terminal end of these peptides (Fig. 3).

The *dsrA* and *dsrB* encoded peptides were also found to contain cysteine arrangements similar to those of [Fe₄S₄] ferredoxins (George *et al.*, 1985), where they are known to participate in the binding of [Fe₄S₄] clusters (Adman *et al.*, 1973, 1976). These sequences occur just beyond the second group of putative sirohaem-binding cysteines in the *dsrA* and *dsrB* encoded peptides. The ferredoxin-like region in the *dsrA* encoded peptide differs from the typical sequence Cys-X₂-Cys-X₂-Cys-X₃-Cys (George *et al.*, 1985) by substitution of Met for the final Cys. However, in all ferredoxin sequences studied so far it seems that the requirement for binding [Fe₄S₄] is the presence of Cys-X₂-Cys-X₂-Cys, either preceded or followed by a distant Cys-Pro sequence (Adman *et al.*, 1976; George *et al.*, 1985; Bruschi & Guerlesquin, 1988). These requirements are met in the *dsrB* as well as in the *dsrA* derived sequence, where Cys-Pro precedes Cys-X₂-Cys-X₂-Cys by either 21 or 19 residues. The arrangement of Cys residues in the *dsrA* encoded peptide is comparable to that found in ferredoxins from *Desulfovibrio africanus* (Bruschi & Hatchikian, 1982) and *Bacillus stearothermophilus* (Hase *et al.*, 1976). By analogy with these two sequences and with the three-dimensional structure of *Peptococcus aerogenes* ferredoxin (Adman *et al.*, 1973, 1976), we propose that [Fe₄S₄] clusters in the *A. fulgidus* sulphite reductase are linked to Cys266, Cys285, Cys288 and Cys291 in the *dsrA* encoded peptide and to Cys219, Cys240, Cys243 and Cys246 in the *dsrB* encoded peptide.

Discussion

A sirohaem-[Fe₄S₄] containing protein exhibiting features specific for dissimilatory sulphite reductases was purified from the extremely thermophilic archaeon *A. fulgidus*. Furthermore we cloned and sequenced *dsrA* and *dsrB*, the genes encoding the α and β subunits of this enzyme. The archaeal sulphite reductase revealed strong similarities with its bacterial counterparts with respect to molecular mass, subunit composition and content of prosthetic groups. It may therefore be stated that the central role of this enzyme in dissimilatory sulphate reduction is reflected in a highly conserved protein structure.

A. fulgidus sulphite reductase and sulphite reductases from dissimilatory sulphate-reducing bacteria in general are found in the soluble, non-membrane fraction after cell disruption (LeGall & Fauque, 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 sulphite reductase, and it has been suggested that the enzyme may be membrane associated *in vivo* (Drake & Akagi, 1977). A hydropathy analysis profile (Kyte & Doolittle, 1982) of the *dsrA* and *dsrB* encoded peptides (not shown) did not reveal markedly hydrophobic segments that would be likely to mediate membrane integration. This however, does not exclude reaction of the soluble sulphite reductase with membrane-bound electron carriers. Possible mechanisms include direct interactions with an integral membrane protein analogous to the reaction of highly water-soluble, periplasmic cytochrome c_2 with the photosynthetic reaction centre in some purple non-sulphur bacteria (Amesz & Knaff, 1988). Alternatively, other redox proteins could be involved in electron flow from membrane-bound carriers to sulphite reductase, analogous to c -type cytochromes which can mediate electron transport from ubiquinol to soluble, periplasmic reductases like nitrite, nitrous oxide or TMAO reductases (Ferguson *et al.*, 1987).

The finding that the peptides encoded by *dsrA* and *dsrB* both contain sequences homologous to putative sirohaem-[Fe₄S₄] binding sites (Ostrowski *et al.*, 1989*b*) was unexpected, since quantitative measurements showed the presence of only 2 mol sirohaem per mol of the $\alpha_2\beta_2$ -structured *A. fulgidus* sulphite reductase. Most other dissimilatory-type sulphite reductases have also been reported to contain only two sirohaem units per $\alpha_2\beta_2$ molecule (LeGall & Fauque, 1988). It may be significant in this context that the *dsrB* peptide contains a Thr in place of the first Cys residue in the Cys-X₅-Cys motif and a Cys in place of the strictly conserved Gly preceding the Cys-X₃-Cys motif (Fig. 3). These changes may reflect an inability of the β subunit to bind sirohaem-[Fe₄S₄], which would account for the observed stoichiometry. Alternatively, the sirohaem-[Fe₄S₄] binding site in each subunit may function as a half site in the $\alpha_2\beta_2$ enzyme, as has been observed for FAD and FMN binding by the flavoprotein component of *E. coli* NADPH-sulphite reductase (Ostrowski *et al.*, 1989*a*).

The chemically determined content of iron and acid labile sulphide indicates that the native sulphite reductase from *A. fulgidus* contains six [Fe₄S₄] clusters per $\alpha_2\beta_2$ molecule. The nature of the iron cores is suggested by preliminary electron paramagnetic resonance spectroscopic evidence (J. Lampreia, unpublished). The EPR spectrum of the oxidized protein showed signals typical for [Fe₃S₄] clusters which probably originate from *in vitro* destruction/conversion of some [Fe₄S₄] clusters as observed for APS reductase from *A. fulgidus* (Lampreia *et al.*, 1991). The presence of [Fe₄S₄] centres in *A. fulgidus*

sulphite reductase is furthermore supported by the occurrence of amino acid sequences characteristic for [Fe₄S₄]-ferredoxins. We propose that two of the [Fe₄S₄] cores are present as sirohaem-[Fe₄S₄] clusters, while the other four would be bound by the ferredoxin-like sequences in the α and β subunits.

To our knowledge, it has never been proposed that the two subunits of dissimilatory sulphite reductases may be related. However, the *dsrA* and *dsrB* encoded peptides show a high degree of similarity and it therefore seems likely that these genes arose by duplication of an ancestral gene. The sirohaem content of the *A. fulgidus* enzyme suggests that one of the subunits may have lost the ability to bind this cofactor, perhaps as a result of changes in the *dsrB* encoded peptide as discussed earlier. It will be interesting to see whether the genes encoding the non-identical subunits of other dissimilatory type sulphite reductases are also homologous to each other or if this is a unique feature of the *A. fulgidus* enzyme.

Among dissimilatory sulphate-reducing bacteria, the extremely thermophilic species of the genus *Thermodesulfobacterium* are the most closely related to the Archaea (Devereux *et al.*, 1989). If sulphite reductase genes have arisen from a common ancestor, the enzymes from these species would be expected to be more closely related to the archaeal sulphite reductase than those from any other bacteria. Indeed, sulphite reductases from *T. mobilis* and *T. commune* share many similarities with the *A. fulgidus* enzyme. An amino acid analysis of the purified enzyme from *Thermodesulfobacterium mobilis* (formerly called *Desulfovibrio thermophilus*; Rozanova & Pivovarova, 1988) showed a cysteine content of 52 residues per 175 kDa tetramer (Fauque *et al.*, 1990), which is identical to that predicted for the $\alpha_2\beta_2$ holoenzyme from *A. fulgidus*. In addition, we found that antibody to the *A. fulgidus* enzyme cross-reacts with a highly purified preparation of the enzyme from *T. commune* (Hatchikian & Zeikus, 1983) in immunoblots of SDS-PAGE gels (not shown). It is interesting to note that the enzymes from these two organisms contain four sirohaem units per $\alpha_2\beta_2$ molecule (Hatchikian & Zeikus, 1983; Fauque *et al.*, 1990). Furthermore, the *T. mobilis* sulphite reductase has been reported to contain eight [Fe₄S₄] clusters (Fauque *et al.*, 1990) compared to the six we find in the *A. fulgidus* enzyme. It seems likely that each subunit of the *T. mobilis* sulphite reductase binds one sirohaem-[Fe₄S₄] prosthetic group and one additional [Fe₄S₄] centre. We predict that the α and β subunits of this enzyme will prove to be homologous and that, in contrast to the *dsrB* encoded peptide, both will contain 'complete' sirohaem-[Fe₄S₄] binding motifs.

Amino acid sequence data has been published for only one other dissimilatory type sulphite reductase. Comparison of the deduced *dsrA* peptide with the chemically

Fig. 4. Organization of homologous elements in sirohaem-[Fe₄S₄] binding proteins. Dashed lines mark gaps that were introduced in order to align all homologous regions. Abbreviations, see Fig. 3.

determined amino acid sequence of the α subunit of *D. vulgaris* dissimilatory sulphite reductase (Pierik *et al.*, 1992) showed identities of 15 of the 25 amino acids sequenced, indicating that these two peptides are homologous (not shown). Significant identities were not found between *A. fulgidus* deduced sequences and the amino-terminal sequences of the β and the recently discovered γ subunit of the *D. vulgaris* enzyme, and additional sequence data from the latter two peptides will be required to determine their relatedness to the *A. fulgidus* peptides.

The *dsrA* and *dsrB* encoded peptides and the *asrC* encoded subunit of the anaerobically expressed sulphite reductase from *S. typhimurium* (Huang & Barrett, 1991) differ from other sirohaem-binding proteins by the presence of a ferredoxin-like domain located just beyond the putative sirohaem-[Fe₄S₄] binding cysteines. Unlike the *asrC* encoded peptide, the *dsrA* and *dsrB* deduced sequences include only one instead of two probable [Fe₄S₄] binding sites. Fig. 4 gives a schematic representation of the distribution of homologous elements in sirohaem-[Fe₄S₄] binding proteins. Introduction of a gap into the sequences of the six sirohaem-binding proteins that do not contain ferredoxin-like sequences gives an alignment that shows regions of homology on both sides of the ferredoxin-domains of *dsrA*, *dsrB* and *asrC*. This finding leads us to propose that a common ancestral gene existed for a sirohaem-[Fe₄S₄] binding reductase and that the ferredoxin-like domain in the *dsrA*, *dsrB* and *asrC* encoded peptides may have resulted from the insertion of a ferredoxin gene into this gene. This hypothesis is supported by the fact that the ferredoxin-like domains in the *dsrA*, *dsrB* and *asrC* encoded peptides are approximately 60 amino acids in length, which corresponds to the size of ferredoxins (George *et al.*, 1985; Bruschi & Guerlesquin, 1988). The

postulated insertion event probably occurred before the divergence of Archaea and Bacteria, since the ferredoxin-like sequences are located at precisely the same position in the sirohaem-[Fe₄S₄] domain in both types of organisms. The same reasoning applies to conclude that the gene duplication event that led to the two homologous peptides of the archaeal sulphite reductase took place after the insertion of the ferredoxin domain. The hypothetical ancestor of all sirohaem-[Fe₄S₄] binding domains could resemble the low-spin sulphite reductase from *D. vulgaris*, a small monomeric enzyme lacking a ferredoxin domain. It is tempting to speculate that all sequences encoding those sirohaem-binding domains without ferredoxin-like sequences originate directly from this ancestral gene and other domains were added during evolution. These domains now bind additional prosthetic groups (e.g. FAD) in the more complex enzymes like the nitrite reductases from *E. coli* and *Aspergillus nidulans*.

Amino acid comparisons of proteins with conserved functions provide a powerful tool for detecting evolutionary relatedness. Sirohaem-binding proteins appear to be well-suited for those studies and the comparisons presented here allowed first glimpses into the evolution of this class of proteins. Future work will focus on gaining information about the evolution of dissimilatory sulphite reductases by comparative sequence analysis of the respective proteins from closely and distantly related, thermophilic and mesophilic organisms.

We thank Karl O. Stetter for kindly supplying us with frozen cell material. Support of this work by grant Tr 133/20-1 from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie is gratefully acknowledged. We wish to thank Hans P. Weil for help with the production of antiserum. C. Dahl was supported by a scholarship of the Studienstiftung des Deutschen Volkes. C. Dahl wishes to thank N. M. Kredich for help and hospitality extended to her. This work was supported in part by research grant DK-12828 (to N.M.K.) from the National Institute of Health.

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