A dissimilatory sirohaem-sulfite-reductase-type protein from the hyperthermophilic archaeon *Pyrobaculum islandicum*

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A sulfite-reductase-type protein was purified from the hyperthermophilic crenarchaeote *Pyrobaculum islandicum* grown chemoorganoheterotrophically with thiosulfate as terminal electron acceptor. In common with dissimilatory sulfite reductases the protein has an $\alpha_2\beta_2$ structure and contains high-spin sirohaem, non-haem iron and acid-labile sulfide. The oxidized protein exhibits absorption maxima at 280, 392, 578 and 710 nm with shoulders at 430 and 610 nm. The isoelectric point of pH 8.4 sets the protein apart from all dissimilatory sulfite reductases characterized thus far. The genes for the α - and β -subunits (*dsrA* and *dsrB*) are contiguous in the order *dsrAdsrB* and most probably comprise an operon with the directly following *dsrG* and *dsrC* genes. *dsrG* and *dsrC* encode products which are homologous to eukaryotic glutathione S-transferases and the proposed γ -subunit of *Desulfovibrio vulgaris* sulfite reductase, respectively. *dsrA* and *dsrB* encode 44.2 kDa and 41.2 kDa peptides which show significant similarity to the two homologous subunits DsrA and DsrB of dissimilatory sulfite reductases. Phylogenetic analyses indicate a common protogenetic origin of the *P. islandicum* protein and the dissimilatory sulfite reductases from sulfate-reducing and sulfide-oxidizing prokaryotes. However, the protein from *P. islandicum* and the sulfite reductases from sulfate-reducers and from sulfur-oxidizers most probably evolved into three independent lineages prior to divergence of archaea and bacteria.**

Keywords: *Pyrobaculum islandicum*, hyperthermophilic archaeon, dissimilatory sirohaem sulfite reductase, nucleotide sequence, evolution

INTRODUCTION

Since the discovery of dissimilatory sulfur reduction in *Desulfuromonas acetoxidans* (Pfennig & Biebl, 1976) many sulfur-respiring prokaryotes have been described, most of which are hyperthermophilic archaea (Widdel, 1988; Stetter *et al.*, 1990; Adams, 1990; Fauque *et al.*, 1991; Schauder & Kröger, 1993). Some of these sulfur respirers are able to use sulfite or thiosulfate as

additional electron acceptors (Widdel & Hansen, 1992; Schauder & Kröger, 1993). The hyperthermophilic crenarchaeote *Pyrobaculum islandicum*, an organism with an optimum growth temperature of 100 °C which was isolated from an Icelandic geothermal power plant (Huber *et al.*, 1987), is a representative of this group. Elemental sulfur is essential as electron acceptor for chemolithotrophic growth of this organism on H₂; however, during organotrophic growth *P. islandicum* is also able to grow on sulfite, thiosulfate, cystine and oxidized glutathione as hydrogen acceptors. *P. islandicum* is not able to reduce sulfate.

While a reasonable number of studies have focused on the metabolism of elemental sulfur in archaea (Ma *et al.*, 1993; Kletzin, 1994; Pedroni *et al.*, 1995; Ma & Adams,

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1994) the enzymes catalysing the reduction of thiosulfate and sulfite in these organisms have hardly been studied. Sulfite reductase from the dissimilatory sulfate reducer *Archaeoglobus fulgidus* is the only enzyme involved in sulfite reduction that has so far been characterized from an archaeon (Dahl *et al.*, 1993).

Sulfite reductase (EC 1.8.99.1) catalyses the six-electron reduction of sulfite to sulfide and is present in all organisms capable of reducing sulfite during anaerobic respiration investigated thus far (Fauque *et al.*, 1991; Dahl *et al.*, 1993). In addition, sulfite reductases with a proposed function in sulfide oxidation have been demonstrated for *Thiobacillus denitrificans* RT and *Chromatium vinosum* strain D (Schedel & Trüper, 1979; Schedel *et al.*, 1979; Hipp *et al.*, 1997). Although several types of dissimilatory sulfite reductases can be differentiated on the basis of their spectral and redox properties, all of these enzymes share common characteristics: they consist of two different polypeptides in an $\alpha_2\beta_2$ structure and contain sirohaem, non-haem iron and acid-labile sulfide (Peck & Lissolo, 1988; LeGall & Fauque, 1988; Fauque *et al.*, 1991). In addition, the two primary sequences available from dissimilatory sulfate reducers (Dahl *et al.*, 1993; Karkhoff-Schweizer *et al.*, 1995) and the amino acid sequence of the sulfite reductase from *C. vinosum* show remarkable similarity, which led to the proposal that these enzymes have a common protogenetic ancestor (Hipp *et al.*, 1997).

In this study we intended to clarify whether a protein similar to sirohaem sulfite reductase is present in thiosulfate- and sulfite-reducing prokaryotes not capable of sulfate reduction. We chose *P. islandicum* as an example because detection of the protein in this organism and sequencing of the respective genes allowed us to extend comparative analyses and phylogenetic considerations to a sulfite reductase of crenarchaeal origin.

METHODS

Bacterial strains, plasmids and media. *P. islandicum* (DSM 4184^T) was grown with 4 mM sodium thiosulfate as electron acceptor as described by Huber *et al.* (1987). Mass cultures for enzyme production were grown in 300 litre enamel-protected fermenters which were gassed with N₂. At 96 °C, the organisms were allowed to grow for 88 h, harvested and stored as packed deep-frozen cells. *Escherichia coli* XL1-Blue (*endA1 supE44 hsdR17 thi relA1 recA1 gyrA46 lac F'* [*proAB⁺ lacI^a lacZΔM15 Tn10 (tet^r)*]) was the host for λ ZAP II (Stratagene) and pBluescript plasmid derivatives. *E. coli* DH5 α [*supE44 ΔlacU169 (φ80dlacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] and *E. coli* NM522 [*supE thi Δ(lac-proAB) hsdS F'* (*proAB⁺ lacI^a lacZΔM15*)] were the hosts for pUC18 (Yanisch-Perron *et al.*, 1985) derivatives and exonuclease-III-generated plasmids, respectively. *E. coli* strains were grown in LB medium (Sambrook *et al.*, 1989) or in double-strength YT medium (Miller, 1972). The media were supplemented with 12.5 µg tetracycline ml⁻¹ for *E. coli* XL1-Blue and 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.

X-Gal and IPTG were included in solid media to identify recombinant phage and plasmids containing inserts in the α portion of *lacZ*.

Assays and spectroscopic measurements. During purification procedures the sulfite-reductase-type protein from *P. islandicum* was followed by measuring the absorbances at 394 and 578 nm and the enrichment was monitored by the A_{280}/A_{578} ratio. Concentrations of the protein are based on a molecular mass of 170 800 Da, which was calculated from the deduced amino acid sequences 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 BSA as a standard. Non-haem iron content was determined by the method of Massey (1957). Acid-labile sulfide was determined according to King & Morris (1966). Sirohaem content was measured by the method of Siegel *et al.* (1978).

Protein purification. Unless otherwise noted all purification steps were performed aerobically at room temperature in 20 mM Tris/HCl, pH 9.5. Packed cells were taken up in about three times their volume of standard buffer, and disrupted by ultrasonic treatment (Schöller disintegrator, 1 min ml⁻¹ for 20 s intervals at 4 °C with maximum power). The broken cell mass was then centrifuged at 25 000 g for 30 min to remove larger fragments. Ammonium sulfate was added to the supernatant of a subsequent centrifugation at 140 000 g to 40% saturation at 0 °C. The precipitated protein was separated from the supernatant by centrifugation (25 000 g, 30 min) and discarded. The supernatant was applied to a Pharmacia-FPLC system and was separated on a Phenyl-sepharose CL-4B FastFlow (low-substituted) column (volume 90 ml, XK26/60 column) equilibrated with 40% ammonium sulfate in standard buffer. Using a linear decreasing gradient from 40% to 0% ammonium sulfate (700 ml) the sulfite-reductase-type protein eluted between 5% and 0% ammonium sulfate. The combined fractions containing the enzyme had a purity index of 22.2 (A_{280}/A_{578}) and were dialysed against standard buffer with 150 mM NaCl after concentration by ultrafiltration using Amicon XM 50 or YM 30 filters. The protein solution was subjected to gel filtration on a HiLoad 16/60 Superdex 200 column equilibrated with standard buffer containing 150 mM NaCl at a flow rate of 0.2 ml min⁻¹. The combined fractions (purity index 13.4) containing the protein were dialysed against start buffer (25 mM Tris/acetate, pH 8.8), and portions of less than 10 mg total protein were further purified by chromatofocusing on Mono P HR 5/20, equilibrated with the same buffer. After washing the column with several volumes of start buffer to elute non-binding proteins, a pH gradient from pH 8.5 to pH 7.5 was applied using Pharmalyte and Polybuffer 96 (Pharmacia) as indicated by the supplier. The green protein was recovered at pH 8.4 and separated from the ampholytes via gel filtration on Superose 6 equilibrated with standard buffer containing 150 mM NaCl. Approximately 3 mg electrophoretically homogeneous protein with a purity coefficient of 6.8 were obtained per 10 g cells.

Chemical and immunological characterizations of the protein. Non-denaturing PAGE was carried out with a cathodic buffer system (Reisfeld *et al.*, 1962). The molecular mass of the native enzyme was estimated by running gels with varying acrylamide concentrations after Hedrick & Smith (1968) and analysed 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. SDS-PAGE was carried out as described by Laemmli (1970). For immunoblots, the polypeptides were transferred from polyacrylamide gels to nitrocellulose membranes (Towbin *et*

al., 1979) using a Bio-Rad semi-dry cell. The blots were blocked with 5% skim milk and incubated with antiserum against *A. fulgidus* sulfite reductase induced in rabbits (Dahl *et al.*, 1993). Binding of the primary antibody was detected with horseradish-peroxidase-conjugated secondary antibody and immunoblot staining with 3',3'-diaminobenzidine after Sambrook *et al.* (1989). Protein sequencing was done as described previously (Dahl *et al.*, 1993). The isoelectric point of sulfite reductase was determined by analytical isoelectric focusing on Servalyt Precotes 3-10 using protein mixture 9 from Serva for calibration of the pH gradient.

Recombinant DNA methods. General methods were those described by Sambrook *et al.* (1989) and Ausubel *et al.* (1996). Unsheared genomic DNA of *P. islandicum* was obtained according to the protocol for *A. fulgidus* (Dahl *et al.*, 1993). Exonuclease deletion clones were prepared using the nested deletion kit from Pharmacia. Sequencing was done on both strands by the dideoxy chain-termination method (Sanger *et al.*, 1977) with both universal and *de novo*-synthesized primers using either the silver sequencing kit of Promega or *Taq* DNA polymerase and dye-labelled dideoxynucleotides. Dye-labelled DNA was analysed with a 373A DNA Sequencer from Applied Biosystems.

For DNA amplification by PCR, 25 pmol of each primer, 200 ng chromosomal template DNA, 2 U *Taq* DNA polymerase (Appligene), 5 µl *Taq* DNA polymerase buffer (100 mM Tris/HCl, pH 9.0, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100, 2.0 mg BSA or gelatin) and 0.2 mM of each deoxynucleotide triphosphate were combined in a final reaction volume of 50 µl. After an initial denaturation step at 95 °C for 5 min, amplifications were carried out for 35 cycles, with each cycle consisting of 30 s at the appropriate annealing temperature, 30–90 s at 72 °C depending on the length of the product and 30 s at 95 °C, followed by annealing for 30 s and incubation at 72 °C for 5 min. DNA probes for Southern blot experiments and screening of libraries were digoxigenin-labelled and colony hybridizations were performed following the protocol of Boehringer. PCR products were purified by gel electrophoresis and electroelution prior to cloning and hybridization steps. Southern and plaque hybridizations were performed overnight at 68 °C as described previously (Dahl *et al.*, 1994).

Cloning strategy. N-terminal peptide sequences for both the α - and the β -subunit of the sulfite-reductase-type protein could not be obtained, suggesting that they are blocked. Five tryptic peptides for a total of 49 residues and three tryptic peptides for a total of 38 residues were sequenced from the α - and β -subunits, respectively. Since the localization of the tryptic peptides within one subunit was not known, four sets of degenerate oligonucleotides were derived from the two chemically sequenced tryptic peptides of the α -subunit FIGTWK and WFVGYL covering the two possible orientations. The four oligonucleotide mixtures were used as primers for PCR in the two possible combinations with genomic *P. islandicum* DNA as the template. A 300 bp PCR product was cloned in the pGEM-T vector (Promega) and sequenced. The derived amino acid sequence showed 33% identity to amino acids 235–331 of the α -subunit of sulfite reductase from *A. fulgidus*. The 300 bp amplicon was used as a probe to screen a λ ZAP II library of 5.5–7.5 kb *Bgl*II fragments of *P. islandicum* DNA. This library was constructed by digestion of *P. islandicum* DNA with *Bgl*II, filling in the restriction sites with the Klenow fragment of *E. coli* DNA polymerase and blunt-end ligation of the DNA to a *Not*I/*Eco*RI adapter. Size-fractionated fragments of 5.5–7.5 kb were ligated into λ ZAP II, which had been digested with *Eco*RI and dephosphorylated.

pBluescript derivatives were prepared from λ ZAP II by using the R408 single-stranded helper phage according to the protocol provided by Stratagene. Each of the ten positive clones analysed was found to contain the same 6.5 kb insert. Restriction enzyme and Southern hybridization analyses located the 300 bp fragment on a central 1 kb *Hind*III/*Bst*XI fragment flanked by 2 and 3 kb DNA portions of the insert. A 3229 bp portion of the cloned *Bgl*II fragment was sequenced on both strands using a combination of subcloning via restriction enzyme or exonuclease III digestion and primer walking. The sequence data indicated the presence of a truncated ORF with homology to *dsrC* from *Desulfovibrio vulgaris* (Karkhoff-Schweizer *et al.*, 1993) at the 3' end of the sequenced DNA. A 225 bp PCR amplicon spanning bases 3003–3328 of the sequenced DNA fragment was used as a probe to identify several positive clones with a 1.5 kb *Hind*III/*Eco*RV insert containing the gene in full in a library of 1.2–1.7 kb *Hind*III/*Eco*RV fragments in the pUC18 vector.

RESULTS

Purification and physico-chemical characterization of a sirohaem-sulfite-reductase-type protein from *P. islandicum*

In all prokaryotes tested so far, growth on thiosulfate as terminal electron acceptor requires the presence of an enzyme which reductively cleaves thiosulfate to sulfite and sulfide and the presence of a dissimilatory sirohaem sulfite reductase which catalyses the six-electron reduction of sulfite to sulfide (LeGall & Fauque, 1988; Fauque *et al.*, 1991). Likewise, extracts of thiosulfate-grown *P. islandicum* exhibited UV-visible spectra indicative of the presence of a sirohaem-containing protein. Purification from the soluble fraction led to a green, electrophoretically homogeneous protein with an absorption spectrum similar to those obtained for dissimilatory sirohaem sulfite reductases. The protein was shown to constitute 0.8% of the total cellular soluble protein. The relative molecular mass was found to be 170 kDa by gel filtration and 165 kDa by electrophoresis in polyacrylamide gels after Hedrick & Smith (1968). SDS-PAGE analysis revealed that the protein consists of two different subunits of 42 and 40 kDa. Our results indicate that the native protein is a tetramer with a quaternary structure of $\alpha_2\beta_2$. The isoelectric point was determined to be at pH 8.4 by analytical isoelectric focusing.

The oxidized protein exhibited absorption maxima at 281, 394 and 578 nm with a weak and a marked shoulder at 430 and 610 nm, respectively (Fig. 1). Furthermore, highly concentrated protein solutions showed a marked band around 710 nm, indicating the presence of high-spin sirohaem (Stolzenberg *et al.*, 1981), a feature shared by all dissimilatory sulfite reductases investigated thus far (LeGall & Fauque, 1988; Fauque *et al.*, 1991). The enzyme was not reducible with dithionite at room temperature. The molar extinction coefficients of the purified *P. islandicum* protein at 281, 394 and 578 nm were estimated to be 366, 175 and 52 mM⁻¹ cm⁻¹, respectively.

Upon extraction with acetone hydrochloride (Siegel *et al.*, 1978) the haem prosthetic group of the enzyme

Fig. 1. Absorption spectrum of oxidized sulfite-reductase-type protein from *P. islandicum*. The spectrum of a 2.1 μ M protein solution in 20 mM Tris/HCl, pH 9.5, was recorded under nitrogen at room temperature. Inset, 9.8 μ M protein solution.

exhibited absorption peaks at 594 and 365 nm, similar to sirohaem preparations obtained from sulfite reductases (Siegel *et al.*, 1978; Fauque *et al.*, 1991; Dahl *et al.*, 1993). After transfer to pyridine the resulting pyridine

haemochrome yielded a spectrum with maxima at 557 and 399 nm typical for metallated sirohaem (Siegel *et al.*, 1978; Murphy *et al.*, 1974). The intensity of the spectrum corresponded to approximately two sirohaem groups per $\alpha_2\beta_2$ unit. This result is confirmed by the extinction coefficient at the Soret peak, which is very close to that of dissimilatory sulfite reductase from *A. fulgidus*. This enzyme has also been reported to contain two sirohaem groups per molecule (Dahl *et al.*, 1993). An analysis for acid-labile sulfide gave a value of 24 mol sulfide per mol of enzyme. Iron determinations gave a value of 22 non-haem Fe atoms per enzyme molecule.

The molecular mass and quaternary structure, as well as the presence of sirohaem, iron and acid-labile sulfide and the absorption spectrum with a Soret peak centred around 390 nm classify the protein purified from *P. islandicum* as a dissimilatory sulfite reductase (Table 1). However, the protein does not exactly resemble any of the sulfite reductases characterized so far. The spectrum lacks the pronounced absorption peak at 628 nm and the split Soret peak present in desulfovibridin (Lee & Peck, 1971) as well as the maximum absorption at 545 nm shown by desulforubidin (Lee *et al.*, 1973b; Arendsen *et al.*, 1993). The *C. vinosum*, *T. denitrificans* and *A. fulgidus* enzymes have maxima at 595, 594 and 592 nm, respectively, instead of 578 nm (Schedel *et al.*, 1979; Schedel & Trüper, 1979; Dahl *et al.*, 1993). The UV-visible spectrum of the *P. islandicum* enzyme most closely resembles that of P582 from the spore-forming

Table 1. Comparison of the *P. islandicum* sirohaem-sulfite-reductase-type protein with dissimilatory sulfite reductases from other organisms

References: 1, Dahl *et al.* (1993); 2, Hatchikian & Zeikus (1983); 3, Lee *et al.* (1973a); 4, Karkhoff-Schweizer *et al.* (1995); 5, Wolfe *et al.* (1994); 6, Pierik *et al.* (1992); 7, Schedel *et al.* (1979); 8, Hipp *et al.* (1997).

Property	<i>Pyrobaculum islandicum</i>	<i>Archaeoglobus fulgidus</i> ¹	<i>Thermodesulfobacterium commune</i> ² (desulfofuscidin)	<i>Desulfovibrio vulgaris</i> ^{3,4,5,6} (desulfovibridin)	<i>Chromatium vinosum</i> ^{7,8}
Absorption maxima (nm), ϵ (mM ⁻¹ cm ⁻¹)	394, 175; 578, 52	394, 184; 593, 60	389, 310; 576, 89	390, NG; 408, 150; 580, NG; 628, 50	392, 302; 594, 98
Molecular mass (kDa)*	170	218	167	226	280
Subunit composition and molecular mass (kDa)	2 α (44.2)† 2 β (41.2)†	2 α (47.4)† 2 β (41.7)†	2 α (47) 2 β (47)	2 α (49.1) 2 β (42.5) 2 γ (11.9)?	4? α (46.8)† 4? β (40.0)†
Non-haem iron‡	22	22–24	20–21	10–22§	51
Acid-labile sulfur‡	24	20	16–17	10–22	47
Sirohaem‡	2.1	2	4	2	NG
Isoelectric point (pH)	8.4	4.2	<7	4.3–4.5	NG

NG, Not given.

*Determined by gel filtration or equilibrium sedimentation.

†Deduced from nucleotide sequence.

‡Atoms or groups per $\alpha_2\beta_2$ -structured enzyme molecule ($\alpha_4\beta_4$ -structured in the case of *C. vinosum*).

§Total iron.

The entire sequenced DNA fragment has a G+C content of 50.4 mol %, which is slightly higher than the value of 46 % found for *P. islandicum* total DNA (Huber *et al.*, 1987). In common with many other archaeal genes (Brown *et al.*, 1989; Tiboni *et al.*, 1993; Pedroni *et al.*, 1995; Dahl *et al.*, 1993; Speich *et al.*, 1994), *P. islandicum* shows a marked preference for AGG and AGA among the arginine codons. The observed strong preference for C in codons of the type NNC/T may favour efficient translation (Cram *et al.*, 1987). 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.

BLAST (Altschul *et al.*, 1990) and FASTA (Devereux *et al.*, 1984) searches with the amino acid sequences of ORF1 and ORF2 indicated strong similarities with the α - and β -subunits of the dissimilatory sirohaem sulfite reductases from *A. fulgidus*, *D. vulgaris* and *C. vinosum* (Dahl *et al.*, 1993; Hipp *et al.*, 1997). As reported for the sulfite reductase genes from the latter three organisms (Dahl *et al.*, 1993; Karkhoff-Schweizer *et al.*, 1995; Hipp *et al.*, 1997), ORF1 and ORF2 are homologous to each other and probably arose by gene duplication. Weak similarities to other sirohaem-containing proteins (assimilatory sulfite reductase and nitrite reductase) were also found. According to the nomenclature for dissimilatory sulfite reductase genes we named the respective *P. islandicum* ORFs *dsrA* and *dsrB*. Sulfite reductase genes have also tentatively been identified in the course of a genome sequencing project on *Pyrobaculum aerophilum* (Fitz-Gibbon *et al.*, 1997) which is closely related to *P. islandicum*, but in contrast to the latter organism is facultatively aerobic (Völkl *et al.*, 1993). The sequence data are, however, neither complete nor have they yet been deposited in GenBank and are therefore not available for comparative analysis.

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Dissimilatory sulfite reductases are the only sirohaem-containing proteins characterized thus far that contain amino acid sequences typical for the binding of additional [Fe₄S₄] clusters in both subunits (Dahl *et al.*, 1993; Karkhoff-Schweizer *et al.*, 1995; Hipp *et al.*, 1997). *dsrA* and *dsrB* from *P. islandicum* also encode the typical sequence Cys-X₂-Cys-X₂-Cys-X₃-Cys (George *et al.*, 1985), which is located at exactly the same position as in the subunits of the dissimilatory sulfite reductases, namely just beyond the second group of the sirohaem-[Fe₄S₄]-binding cysteines.

ORF3 encodes a 100 aa, 11.5 kDa peptide with a theoretical isoelectric point of pH 4.94 and an aliphatic index of 110.1. Upon FASTA and BLAST searches the ORF3-encoded protein revealed weak but significant

DISCUSSION

A sirohaem-[Fe₄S₄] containing-protein which exhibits features specific for dissimilatory sulfite reductases was purified and characterized from the extremely thermophilic archaeon *P. islandicum*. Furthermore, we cloned and sequenced *dsrA* and *dsrB*, the genes encoding the α - and β -subunits of this protein.

Dissimilatory sulfite reductases are usually assayed with reduced methylviologen as electron donor (Widdel & Hansen, 1992). One way to obtain reduced methylviologen is reduction with hydrogen via hydrogenase, thereby allowing the sulfite reductase reaction to be followed manometrically. As enzymes from the hyperthermophile *P. islandicum* have temperature optima close to 100 °C, the optimum growth temperature of the organism, and a pure, hyperthermophilic hydrogenase was not available, this assay was impracticable. In the case of the hyperthermophilic sulfite reductase from *A. fulgidus* this problem was circumvented by using electrochemically reduced methylviologen and the enzyme could be proven in crude extracts at 85 °C by following the decrease in absorbance at 600 nm (Dahl *et al.*, 1993). However, at 100 °C chemical reoxidation of the dye was so rapid that sulfite reductase activity could not be attributed to the protein from *P. islandicum* purified in this work with absolute certainty.

Nevertheless, several lines of evidence suggest that this protein works as a dissimilatory sulfite reductase in *P. islandicum*. (i) The high concentration of the protein in thiosulfate-grown cells (0.8% of the total cellular soluble protein) indicates a dissimilatory function and is in the same range as found for dissimilatory sirohaem sulfite reductases in sulfate-reducing prokaryotes (Peck & LeGall, 1982; Dahl *et al.*, 1993). (ii) The protein exhibits several characteristics which, to the best of our knowledge, occur exclusively in dissimilatory sulfite reductases: a molecular mass in the range of 200 kDa, an $\alpha_2\beta_2$ -subunit composition, the presence of two sirohaem groups in the high-spin state per holoenzyme, and the presence of 22 non-haem iron atoms and 24 atoms of acid-labile sulfide per holoenzyme, which allow the formation of six [Fe₄S₄] clusters. (iii) Crude extracts of *P. islandicum* and purified protein cross-react with antiserum raised against *A. fulgidus* sulfite reductase (data not shown). (iii) The amino acid sequences of the α - and β -subunits of the *P. islandicum* protein show homology to the respective subunits of dissimilatory sulfite reductases over their entire length. All essential cluster-binding residues are conserved. (iv) Besides sulfite reductases, sirohaem as a prosthetic group has only been described in ammonia-forming assimilatory nitrite reductases. All sirohaem nitrite reductases characterized thus far differ substantially from the *P. islandicum* protein and dissimilatory sulfite reductases with respect to molecular mass, subunit composition, and content of prosthetic groups (Siegel *et al.*, 1987; Romero *et al.*, 1987; Back *et al.*, 1988; Kinghorn & Campbell, 1989; Peakman *et al.*, 1990; Brittain *et al.*, 1992; Lin *et al.*, 1993). The fact that assimilatory nitrite

reductases are usually repressed in the presence of ammonia (Cole, 1988) and the sulfite-reductase-type protein from *P. islandicum* was isolated from cells grown with ammonia as nitrogen source further argues against a function of this enzyme in nitrite reduction. Some sulfate-reducing bacteria can reduce nitrate and contain ammonia-producing nitrite reductases. These enzymes are, however, hexahaem cytochromes (Liu & Peck, 1981). *P. islandicum* is furthermore not able to use nitrate as electron acceptor (Völkl *et al.*, 1993).

It has been suggested that dissimilatory sulfite reductases may be membrane-associated *in vivo* because the generation of a protonmotive force by sulfate or sulfite reduction requires the transfer of reducing power from the membrane-localized electron transport chain to sulfite reductase (Drake & Akagi, 1977). In agreement with this suggestion, Steuber *et al.* (1994) reported the purification of a dissimilatory sulfite reductase from the membrane fraction of *Desulfovibrio desulfuricans*. In contrast, treatment of *P. islandicum* membrane fractions with the detergents SDS, Triton X-114 and CHAPS did not lead to the solubilization of any sirohaem-containing proteins. In this context it may be important to note that after ultracentrifugation Steuber *et al.* (1994) separated whole cells and membrane fractions used to isolate the 'membrane-bound' enzyme only on the basis of a different colour. If whole cells remained in the membrane fraction an extraction with Triton X-100 as applied might well have disrupted these cells. Soluble sulfite reductase present in these cells could then have mimicked the presence of a membrane-bound enzyme. As has been found for all dissimilatory sulfite reductases sequenced so far, hydropathy analyses (Kyte & Doolittle, 1982) of DsrA and DsrB from *P. islandicum* did not reveal markedly hydrophobic segments that would be likely to mediate membrane integration. Rossi *et al.* (1993) proposed that the components of a potential transmembrane redox complex transfer electrons from periplasmic hydrogenase to the soluble cytoplasmic enzymes of sulfate reduction in *D. vulgaris*. A comparable protein complex may donate electrons to the soluble sulfite reductase of *P. islandicum*; it must, however, be borne in mind that the presence of elemental sulfur as electron acceptor is essential for chemoautotrophic growth of the organism on hydrogen (Huber *et al.*, 1987).

Both the *P. islandicum* *dsrA* and *dsrB* genes encode all four cysteine residues required for sirohaem-[Fe₄S₄] binding (Ostrowski *et al.*, 1989; Crane *et al.*, 1995), which suggests the presence of a total of four sirohaem-[Fe₄S₄] groups in the $\alpha_2\beta_2$ -structured holo-protein. However, quantitative measurements unambiguously showed the presence of only two mol sirohaem per mol of holoprotein. With the exception of desulfofusicidin from *Thermodesulfobacterium* species (Hatchikian & Zeikus, 1983; Fauque *et al.*, 1990), all dissimilatory-type sulfite reductases have also been reported to contain only two sirohaem units per $\alpha_2\beta_2$ molecule (LeGall & Fauque, 1988; Dahl *et al.*, 1993; Arendsen *et al.*, 1993). For the dissimilatory sulfite

reductases sequenced so far the presence of only two sirohaem groups has been explained by the mutation, in the β -subunit, of one of the $[\text{Fe}_4\text{S}_4]$ cysteine cluster ligands to a threonine (Dahl *et al.*, 1993). It now seems clear from our results with the *P. islandicum* protein that the presence of all potential cluster-binding cysteine residues cannot be the only prerequisite for sirohaem- $[\text{Fe}_4\text{S}_4]$ binding. In this respect the crystal structure of the *E. coli* assimilatory sulfite reductase haemoprotein (Crane *et al.*, 1995) is very instructive. This protein is proposed to have arisen by fusion of two homologous proteins similar to DsrA and DsrB. Both halves of the assimilatory enzyme fold into similar structures, particularly in the regions that bind the cofactors, suggesting an ancestral homodimer with a sirohaem and an $[\text{Fe}_4\text{S}_4]$ group on each subunit. Subsequently, the protein evolved so that the DsrA-like C-terminal portion of the contemporary protein contains the cysteines that ligate the $[\text{Fe}_4\text{S}_4]$ cluster which is exchange-coupled to the sirohaem group and the DsrB-like N-terminal half contributes most of the residues that bind the sirohaem carboxyl groups. These two parts are joined by a linker region which contributes a short stretch of amino acids that acts as a structural mimic for ligand-bound sirohaem and associates with those C-terminal residues homologous to the sirohaem-carboxylate-binding residues in the N-terminal half of the protein (Crane *et al.*, 1995; Crane & Getzoff, 1996). This stretch of amino acid residues is conserved in the β - but not in the α -subunits of dissimilatory sulfite reductases, and accordingly is found only in DsrB from *P. islandicum* (Fig. 3, homology region H5). If this region indeed forms a structural mimic for sirohaem and occupies the sirohaem-binding site on DsrA only one sirohaem $[\text{Fe}_4\text{S}_4]$ group would be expected per $\alpha\beta$ dimer irrespective of how many potential cluster-ligating cysteines are present in DsrB. In support of this view, Crane & Getzoff (1996) pointed out that it is unlikely that in dissimilatory sulfite reductases one subunit alone can bind both the sirohaem and the $[\text{Fe}_4\text{S}_4]$ cluster and maintain the covalent association of the cofactors indicated by electronic exchange coupling observed between the cofactors.

The chemically determined content of iron and acid-labile sulfide indicates that the sulfite-reductase-type protein from *P. islandicum* contains six $[\text{Fe}_4\text{S}_4]$ clusters per $\alpha_2\beta_2$ molecule. The presence of $[\text{Fe}_4\text{S}_4]$ centres in the protein is supported by the occurrence of amino acid sequences characteristic for $[\text{Fe}_4\text{S}_4]$ -ferredoxins. Similar to the situation in *A. fulgidus*, two of the $[\text{Fe}_4\text{S}_4]$ cores are probably present as sirohaem- $[\text{Fe}_4\text{S}_4]$ clusters, while the other four would be bound by the ferredoxin-like sequences in the α - and β -subunits.

The fact that a gene homologous to *dsuC*, the gene for the proposed 11 kDa γ -subunit of *D. vulgaris* sulfite reductase (Pierik *et al.*, 1992), is found downstream of *dsrAB* in *P. islandicum* was rather unexpected as – comparable to results obtained for the *A. fulgidus* sulfite reductase (Dahl *et al.*, 1993) – SDS-PAGE analyses of the protein purified from *P. islandicum* on high-per-

centage acrylamide gels clearly showed that it does not contain a third subunit. In *D. vulgaris*, the *dsuC* gene is neither linked to nor co-ordinately expressed with those for the α - and β -subunits of sulfite reductase (Karkhoff-Schweizer *et al.*, 1993). In addition, the observation of Steuber *et al.* (1995) that an 11 kDa polypeptide appears to be only loosely associated to *D. desulfuricans* sulfite reductase and is lost in part during purification leads us to doubt that an 11 kDa polypeptide is indeed an integral part of dissimilatory sulfite reductases. However, the observed association of the polypeptide with some sulfite reductases (Pierik *et al.*, 1992; Wolfe *et al.*, 1994; Arendsen *et al.*, 1993), together with the close linkage of the *dsrAB* and *dsrC* genes in *P. islandicum* indicate an important though as yet unknown function of DsrC in sulfite reduction. The presence of a gene homologous to *dsrC* in *E. coli* and *H. influenzae*, organisms which do not contain dissimilatory sulfite reductases but can synthesize assimilatory sirohaem sulfite and nitrite reductases under appropriate growth conditions, may hint at a function of the respective gene products in assembly, folding or stabilization of sirohaem proteins.

Dissimilatory sulfite reductases consist of relatively long polypeptide chains containing both semi-conserved and highly conserved regions, and occur in sulfate- and sulfite-reducing prokaryotes as well as in some sulfur oxidizers (Dahl *et al.*, 1993; Karkhoff-Schweizer *et al.*, 1995; Hipp *et al.*, 1997). Therefore, the sulfite reductase subunits in principle appeared to be suited to trace the evolution of dissimilatory sulfur metabolism. Fig. 6 shows a bootstrap parsimony analysis of an amino acid alignment of the DsrA and DsrB sequences available so far. The tree exhibits a dyad symmetry, i.e. the DsrA and DsrB polypeptides are more closely related among themselves than to each other in one organism. When constant evolutionary rates are assumed this tree topology strongly suggests that the duplication of the common ancestor of the *dsrAB* genes must have happened prior to entering the lineages leading to the contemporary sequences. The observed tree topology deviates from the phylogeny inferred from 16S rRNA-encoding genes which would have placed together the two proteobacteria *C. vinosum* and *D. vulgaris* on one hand and the two archaea *A. fulgidus* and *P. islandicum* on the other (Olsen & Woese, 1994). Instead, the subunits of the *P. islandicum* protein are located on separate branches and the polypeptides from the sulfate reducers *D. vulgaris* and *A. fulgidus* are more closely related to each other than to those of the sulfide-oxidizing *C. vinosum*. As discussed earlier for *C. vinosum*, *D. vulgaris* and *A. fulgidus* (Hipp *et al.*, 1997), the most likely explanation for the observed tree topology is that the sequences compared in Fig. 6 stem from paralogous (Gogarten, 1994) protein families that evolved into independent lineages in a protogenetic (Benner & Ellington, 1990) world, i.e. prior to divergence into Bacteria and Archaea. In addition to the family of sulfite reductases working in the reductive direction and that of the enzymes working in the oxidative direction (Hipp *et al.*, 1997) we now postulate

Fig. 6. Bootstrap parsimony analysis of amino acid alignments of DsrA and DsrB sequences with the *sir* gene product of *D. vulgaris* (Tan *et al.*, 1991) as the outgroup. The unrooted tree was constructed by the program PROTPARS (Felsenstein, 1993) from an alignment produced with the program CLUSTAL W (Higgins & Sharp, 1989) using the Mutation Data Matrix (Dayhoff *et al.*, 1978; George *et al.*, 1990). The alignment comprised only the highly conserved part common to all sirohaem-binding enzymes. Only unambiguously aligned residues as estimated by statistically significant sequence similarities were included for the analyses; gaps in the data sets were avoided. Branch lengths are arbitrary. Numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees out of 1000 bootstrap replications. The tree topology based on protein parsimony was verified by distance matrix analyses. Af, *Archaeoglobus fulgidus*; Cv, *Chromatium vinosum*; Dv, *Desulfovibrio vulgaris*; Pi, *Pyrobaculum islandicum*. Bold characters define archaea.

the existence of a third family of sulfite-reductase-type-proteins, the only currently known representative of which is the enzyme from the crenarchaeote *P. islandicum*. The protogenetic development of at least three different families of sulfite-reductase-type proteins points to an ancient evolutionary origin of the *dsrAB* gene family, which is in accordance with the concept of Skyring & Donnelly (1982) who claimed on the basis of geochemical data that the biochemical evolution of sulfite reduction occurred early on a primordial earth and even was the initial event leading to the evolution of dissimilatory sulfate reduction.

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