Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes

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The genes for adenosine-5'-phosphosulfate (APS) reductase, *aprBA*, and sirohaem sulfite reductase, *dsrAB*, from the sulfur-oxidizing phototrophic bacterium *Chromatium vinosum* strain D (DSMZ 180^T) were cloned and sequenced. Statistically significant sequence similarities and similar physicochemical properties suggest that the *aprBA* and *dsrAB* gene products from *Chr. vinosum* are true homologues of their counterparts from the sulfate-reducing chemotrophic archaeon *Archaeoglobus fulgidus* and the sulfate-reducing chemotrophic bacterium *Desulfovibrio vulgaris*. Evidence for the proposed duplication of a common ancestor of the *dsrAB* genes is provided. Phylogenetic analyses revealed a greater evolutionary distance between the enzymes from *Chr. vinosum* and *D. vulgaris* than between those from *A. fulgidus* and *D. vulgaris*. The data reported in this study are most consistent with the concept of common ancestral protogenetic genes both for dissimilatory sirohaem sulfite reductases and for APS reductases. The *aprA* gene was demonstrated to be a suitable DNA probe for the identification of *apr* genes from organisms of different phylogenetic positions. PCR primers and conditions for the amplification of *apr* homologous regions are described.

Keywords: *Chromatium vinosum*, adenosine-5'-phosphosulfate reductase, sirohaem sulfite reductase, phylogeny, enzyme evolution

INTRODUCTION

The utilization of sulfur compounds for energy-yielding processes seems to have developed very early during prokaryotic evolution (Skyring & Donnelly, 1982; Wächtershäuser, 1988). Prokaryotes still play a dominant role in the oxidative and the reductive phases of the natural sulfur cycle; chemotrophic and phototrophic sulfur- and sulfide-oxidizing prokaryotes produce sulfate, which is used as electron acceptor in dissimilatory sulfate reduction, an anaerobic respiratory process that regenerates sulfide (Trüper & Fischer, 1982; Schidlowski, 1986). Phylogenetic analysis of enzymes participating in dissimilatory sulfate reduction and oxidation of reduced sulfur compounds should

provide new insights into the evolution of dissimilatory sulfur metabolism.

Three enzymes appear to be involved in both the oxidative and the reductive mode of dissimilatory sulfur metabolism: ATP sulfurylase, the iron-sulfur flavo-protein adenosine-5'-phosphosulfate (APS) reductase (EC 1.8.99.2) and sirohaem sulfite reductase (EC 1.8.99.1). All three enzymes are generally found in both sulfate-reducing bacteria and archaea (Thauer, 1989; Fauque *et al.*, 1991; Speich & Trüper, 1988; Dahl *et al.*, 1990). In addition, the presence of APS reductase and ATP-sulfurylase has been reported for several species of chemotrophic (Takakuwa, 1992) and phototrophic (Trüper & Fischer, 1982; Fischer, 1989) sulfur-oxidizing bacteria. The presence of a sirohaem sulfite reductase with a proposed function in sulfide oxidation has been demonstrated for *Thiobacillus denitrificans* strain RT and *Chromatium vinosum* strain D (Schedel & Trüper, 1979; Schedel *et al.*, 1979). Since APS reductase and sulfite reductase furthermore are highly

Abbreviation: APS, adenosine-5'-phosphosulfate.

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conserved proteins in terms of physicochemical properties (Lampreia *et al.*, 1994; Hatchikian, 1994; Brune, 1989), these enzymes appear to be ideal candidates for phylogenetic analyses. The sequences for sulfite reductase and APS reductase from a hyperthermophilic archaeal (*Archaeoglobus fulgidus*) and a mesophilic bacterial (*Desulfovibrio vulgaris*) sulfate reducer (Dahl *et al.*, 1993; Speich *et al.*, 1994; Karkhoff-Schweizer *et al.*, 1995; GenBank accession number Z69372) provided the basis for our work on the evolution of these enzymes, which we now extend to a mesophilic bacterial sulfur oxidizer, the phototrophic bacterium *Chr. vinosum*.

METHODS

Bacterial strains and growth conditions. *Chr. vinosum* strain D (DSMZ 180^T) and *Chlorobium limicola* f. sp. *thiosulfatophilum* strain 6230 (DSMZ 249^T) were cultured in medium 2 (Pfennig & Trüper, 1992) with the pH adjusted to 7.3 and 6.8, respectively. *Ectothiorhodospira mobilis* strain 51/7 (DSMZ 237^T) was grown in the medium described by Imhoff (1992) for extremely halophilic *Ectothiorhodospira* species with 5% (w/v) salinity. *Rhodovulum adriaticum* strain 6 II (DSMZ 2781^T) and *Rhodovulum sulfidophilum* strain W 4 (DSMZ 1374^T) were cultivated in AT medium (Imhoff & Trüper, 1992) with 5% salinity and sodium sulfide concentrations of 1 mM and 2 mM, respectively. All phototrophic bacteria were grown anaerobically and photolithoautotrophically. The purity of cultures was routinely checked by light microscopy and agar dilution series (Pfennig & Trüper, 1992). In addition, the results of heterologous PCR (see Results and Discussion) excluded the possibility of contamination by sulfate reducers. Medium and anaerobic growth conditions for *T. denitrificans* strain RT (DSMZ 807) were essentially as described by Baldensperger & Garcia (1975). *Escherichia coli* XL1-Blue (Stratagene) [*endA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 λ^- recA1 gyrA96 relA1* (*lac*) [*F'*, *proAB lacI^q* Δ M15 Tn10(*tet^R*)] and *E. coli* DH5 α [*F'*, ϕ 80*lacZ* Δ M15 Δ (*lacZ*Y Δ -argF)U169 *recA1 endA1 hsdR17* ($r_K^- m_K^-$) *supE44 λ^- thi-1 gyrA relA1*] were used as host strains for molecular cloning. *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 μ g tetracycline ml⁻¹ for *E. coli* XL1-Blue and with 100 μ g ampicillin ml⁻¹ for plasmid-containing derivatives.

DNA preparation and PCR primers. Chromosomal DNA from *Chr. vinosum* and *Ect. mobilis* was prepared by sarcosyl lysis (Bazaral & Helinski, 1968) and subsequent CsCl gradient centrifugation (Sambrook *et al.*, 1989); chromosomal DNA from *R. sulfidophilum*, *R. adriaticum*, *Chl. limicola* f. sp. *thiosulfatophilum* and *T. denitrificans* was prepared by the CTAB method (Ausubel *et al.*, 1996), followed by CsCl centrifugation. Chromosomal DNA from *A. fulgidus* strain VC16 (DSMZ 4304^T) was prepared according to Dahl *et al.* (1993). For PCR analysis, 20 mg cell paste from *Desulfovibrio gigas* (DSMZ 1382^T) was washed in 100 μ l TE pH 8.0 (Sambrook *et al.*, 1989) and centrifuged in a microcentrifuge tube at 19000 *g* for 20 s. The pellet was resuspended in 100 μ l TE pH 8.0 and incubated in a boiling water bath for 5 min (Kretz *et al.*, 1994). After centrifugation at 19000 *g* for 2 min, 60 μ l of the supernatant was pipetted into another microcentrifuge tube; 1 μ l of this preparation was used as template in PCR. Primers wh5 (5'-GCATTGAAGAGCGAACT-GCTTGCT-3') and wh6 (5'-CAATGGCAGGGTTCCAA-GTACATG-3'), which correspond to nucleotide positions

445–468 and 2540–2563, respectively, of the *apr* locus of *A. fulgidus* (Speich *et al.*, 1994), were used for the generation of the DNA probe Afwh5wh6^{DIG} for APS reductase genes. DNA concentrations were determined by adsorption spectroscopy (Ausubel *et al.*, 1996). Sequences of the degenerate primers for heterologous PCR were as follows, with the nucleotide positions of the respective gene loci given in parentheses: wh53, 5'-TGGGA(G/A)TGCTAC(A/T)(G/C)CTG-3' [*A. fulgidus* (214–230) (Speich *et al.*, 1994), *D. vulgaris* (353–369) (GenBank accession number Z69372), *Chr. vinosum* (211–227) (this work)]; wh54, 5'-C(T/C)GT(G/T)GCCCAGGG(T/C)CT-3' [*A. fulgidus* (735–750), *D. vulgaris* (962–977), *Chr. vinosum* (718–733)]; and wh62, 5'-C(G/T)GTAGTAG(A/T)A(G/T)CC(G/C)GG-3' [*A. fulgidus* (2333–2349), *D. vulgaris* (2614–2630), *Chr. vinosum* (2292–2308)]. The sequencing primers were pUC/M13 forward, 5'-GTAAACGACGGCCAGT-3'; and pUC/M13 reverse, 5'-GAAACAGCTATGACCATG-3'.

Cloning and DNA sequencing. All general cloning techniques and enzymic manipulations were performed according to standard methods (Ausubel *et al.*, 1996). A clone carrying a 5 kb *Hind*III fragment with the complete *aprBA* genes was isolated from a library of 4.5–5.5 kb *Hind*III fragments of total *Chr. vinosum* DNA in the pUC18 vector using the PCR-generated 400 bp portion of the *Chr. vinosum* APS reductase gene locus described by Dahl (1996) as a probe. For cloning of the *dsr* locus, a library of 3.0–3.9 kb *Eco*RI fragments in pUC18 was constructed. Identification of positive clones was achieved by Southern hybridization at 63 °C of *Eco*RI-restricted plasmid DNA isolated from randomly picked colonies using a 521 bp PCR-generated probe encoding the sirohaem-[Fe₄S₄] binding domain of the α -subunit of *D. simplex* (DSMZ 4141^T) dissimilatory sulfite reductase (Molitor, 1996; GenBank accession number U78738; positions 342–852). Preliminary sequencing indicated that the cloned 3.1 kb fragment did not contain the 5'-terminus of the *Chr. vinosum dsrA* gene. Therefore, the remaining portion of the gene was cloned by chromosome walking on an overlapping 4 kb *Sal*I genomic DNA fragment, which was isolated from a library of 3.5–4.5 kb *Sal*I fragments in the pBluescript II SK(+) vector (Stratagene). To facilitate further manipulations the inserts were recloned into the appropriate sites of pGEM-7Zf(+) (Promega). Deletion clones were constructed by subcloning of restriction fragments or by unidirectional digestions with exonuclease III (Henikoff, 1984), using the Erase-a-base system (Promega) and following the manufacturer's instructions. Double-stranded DNA sequencing was performed with the Silver Sequence system (Promega), following the manufacturer's instructions except that, for both pUC/M13 forward and reverse primers (see above), the PCR profile 1 with an annealing temperature of 49 °C was used.

Southern hybridizations. Hybridizations were performed with the digoxigenin (DIG) system (Boehringer) according to the manufacturer's instructions with the following modifications. The prehybridization and hybridization buffers were supplemented with 0.1 mg sonicated herring sperm DNA (Promega) ml⁻¹. The SDS concentration in the wash solutions was 1% (w/v). The chemiluminescent substrate CSPD (Promega) was applied to a final concentration of 25 μ M. Other modifications and specifications were as described elsewhere (Hipp, 1996). Probes were labelled with digoxigenin via PCR (Lion & Haas, 1990) with final concentrations of 20 μ M DIG-11-dUTP (Boehringer) and 180 μ M dTTP in the reaction mixture; other conditions were as described

below. After purification by agarose gel electrophoresis, the gel slice (50–100 mg) containing the labelled amplicon was excised and transferred to a plastic tube filled with 10 ml hybridization buffer. Incubation for 20 min in a boiling water bath yielded the ready-to-use hybridization solution.

PCR. Hot start PCR (Chou *et al.*, 1992) was performed in a TRIO-Thermoblock thermal cycler (Biometra). Buffer A (10× concentration) consisted of 500 mM KCl, 100 mM Tris/HCl pH 8.3, 15 mM MgCl₂ and 0.1% (w/v) gelatin (Lion & Haas, 1990). The composition of 10× buffer B ('SuperTaq buffer', Stehelin & Cie AG) was 500 mM KCl, 100 mM Tris/HCl pH 9.0, 15 mM MgCl₂, 0.1% gelatin and 1% (v/v) Triton X-100. Reaction mixtures (50 µl) contained 50–250 ng template DNA, 2 U Taq polymerase, 1× PCR buffer, 200 µM of each dNTP and 1 µM of each primer. The temperature profile for the amplification of Afwh5wh6^{DTG} started with a 5 min denaturation step at 95 °C. Cycling was 20 s at 55 °C, 60 s at 72 °C and 30 s at 95 °C for 35 cycles followed by an additional annealing step and a final 5 min elongation step at 72 °C. The thermal profile for the use of degenerate primers consisted, after an initial 3 min denaturation step at 95 °C, of three cycles I (30 s at annealing temperature I, 0.2 °C s⁻¹ ramp time to reach 72 °C, 1 min at 72 °C, 30 s at 95 °C) and 30 cycles II (30 s annealing temperature II, 1 min at 72 °C, 30 s at 95 °C), followed by an additional annealing step at annealing temperature II and a final 5 min elongation step at 72 °C. Annealing temperature I was either 60 °C (Tdwh54wh62) or 50 °C (all others); annealing temperature II was either 50 °C (Tdwh54wh62) or 40 °C (all others). With DNA from *Chl. limicola* f. sp. *thiosulfatophilum* and *T. denitrificans* buffer A was used, while with all other templates buffer B gave better results.

RESULTS AND DISCUSSION

Description of the *apr* locus from *Chr. vinosum*, and alignment with sequences from *A. fulgidus* and *D. vulgaris*

In a BLASTX analysis (Gish & States, 1993; Altschul *et al.*, 1990; blast@ncbi.nlm.nih.gov) a 2567 bp DNA region containing the *apr* locus from *Chr. vinosum* revealed high statistical similarities with smallest sum probabilities equal or less than 1.8×10^{-35} to sequences encoding the subunits of the APS reductases from *A. fulgidus* and *D. vulgaris*. These similarities correspond to two ORFs whose termination and ATG initiation codons overlap in the sequence TGATG. The shorter ORF (nt 85–567) was found to encode a 160 aa polypeptide with a computed molecular mass of 18175 Da. Translation of the larger ORF (nt 567–2429) yields a 620 aa polypeptide with a computed molecular mass of 69543 Da. The identification of the two ORFs as the genes *aprB* and *aprA* for two different subunits of the APS reductase from *Chr. vinosum* is strongly supported by the fact that insertional inactivation of *aprBA* in *Chr. vinosum* leads to an inability of the organism to synthesize APS from sulfite and AMP (Dahl, 1996). Putative ribosome-binding sites (RBS) (AAGAGG, AGCAGG) are properly located immediately upstream of each of the two ORFs. Whilst a promoter-like structure was not evident within the 84 bp upstream of the putative initiation codon of *aprB*, an inverted repeat which gives rise to a stable stem-loop

structure ($\Delta G = -35.0$ kJ mol⁻¹, identified and calculated by DNASIS, Hitachi) immediately followed by a poly(dT) stretch, typical for a rho-independent terminator (Richardson, 1993), is located 28 bp downstream of the *aprA* stop codon.

For greater clarity, in the following text the sequences from *D. vulgaris* are also named *apr* instead of *aps* (GenBank accession number Z69372). Alignments of the deduced amino acid sequences from the respective genes from *A. fulgidus*, *D. vulgaris* and *Chr. vinosum* show regions of statistically significant similarity with a large number of conserved residues spanning almost the entire polypeptides (Fig. 1a, b). Moreover, common secondary structures as predicted by several programs are found even in regions that exhibit no statistically significant sequence similarities.

With respect to the special molecular characteristics of APS reductases, Speich *et al.* (1994) found a high sequence similarity between the flavoprotein subunit (AprA) of APS reductase from *A. fulgidus* and succinate dehydrogenases and fumarate reductases. To verify this result, a BLOCKS search was performed to identify short multiply aligned ungapped segments corresponding to the most highly conserved regions of proteins (Henikoff & Henikoff, 1994; blocks@howard.fhcrc.org). With AprA from *A. fulgidus*, six of eight blocks of the PROSITE (Bairoch, 1992) entry PS00504 ('fumarate reductase/succinate dehydrogenase FAD-binding site proteins') were recognized. The respective polypeptides from *Chr. vinosum* and *D. vulgaris* seemed to be less related to this protein family, as only four of eight blocks scored highly enough to be reported. Nevertheless, the general assignment of the flavoprotein subunits of APS reductases to the aforementioned protein family justified the extension of a comparative analysis to the new data. All essential predictions made by Speich *et al.* (1994) concerning functionally important regions are supported by our findings. For clarity only the positions in the sequences of the polypeptides from *Chr. vinosum* are given here. The corresponding positions in their homologues can be inferred from Fig. 1.

In addition to the segments described by Speich *et al.* (1994), a long carboxy-terminal stretch appears to be functionally important for FAD binding. This stretch comprises residues 492–581 and it shows statistically significant sequence similarities with the flavoprotein subunits of fumarate reductases, succinate reductases and L-aspartate oxidases (double-underlined in Fig. 1b). Robinson & Lemire (1996) removed the 70 carboxy-terminal residues of the flavoprotein subunit of succinate dehydrogenase from *Saccharomyces cerevisiae*. The residual polypeptide was no longer able to bind FAD.

The centre of AprA contains a strictly conserved undecapeptide (residues 247–257). Due to its agreement with the 'core' fingerprint for ADP-binding folds (Wierenga *et al.*, 1986) and the structural similarity of ADP and APS, Speich *et al.* (1994) predicted that this region is part of the APS-binding site. The replacement of a neutral hydrophobic residue by a charged residue

(a) AprB

	t	hheee	hhhhh	eeeeee	FFFFF	
	MP V CDGC	C ICP D M D	AYN P CWECYSCVK CP	I R Y D P G	R	I W
Af	MPSFVNPERCDGC	KALETACEYICPNDLMTLDKEMKAYNRDPDM	DEYEVKMCPOGGQIDVRGYVDYSPLGGACVVRGTS	IMWTDK	91	
Dv	MPTYVDPSKCDGC	KGGENTACMYICPNDLMTLDPEEMRAYNQEPAC	DEYEVKMCPOGGQIDVRGYVDYSPLGGACVVRGTS	IMWTDK	91	
Cv	MPTFYVMTCDGC	-----GQCVDICPSDIMEHIDTIRRAYNIEPNM	DEYEVKMCPOGGQIDVRGYVDYSPLGGACVVRGTS	IMWTDK	88	
	t	EEEEEE	hhhhhhhhh	hhh		
	RNG	I T P G P	S LL E			
Af	YRNG	KVLRFKFAIRTTTPWGSIQPFEGFP	TEELKSELLAGEPEIIGTSEFPQVKKKA	-----	150	
Dv	FRNG	NVRKFPFIRTTTPWGSIKPFEGKPEA	-----GDLSELLFTETALTAPKEALGKFDVTD	DAETSQCWYETGCQGGNR	167	
Cv	FRNG	KDMNLLAPITTKPWGQCTPKLKEVP	PSNEMRDSQLLFNEPKYIRLDDGGLETLESNGLKMQAGVCY	-----	160	

(b) AprA

	eeeeeeFFFFee	t	hhhhhhhhh	eeehhhhhhhhhhhhhhhhhhhhhhhhhhhhh	t	
	D L G G G A E A W	K	KA	RSGAVAQGL AIN Y	N	
Af	MVYYPKYEY-KADEVP-TEVETDILITGGGFCGCAAYEAYWAKVGG	-----LKLTLVEKAAVERSGCAQCHSAINTYIDLTGRSE	ONTL	89		
Dv	MPMIPVKEQPKGVAIAEPTVKEEDVLLIVGGMGACGTAFAEVRWADNYAP	ELKILLIDKASLERSGCAQCHSAINTYLG	-----KND	86		
Cv	-----MAYKTIIEEDIDVLVVGAGLGGTGAFAEARYWGD	-----KIVIAEKANIDRSGCAQCHSAINTYLG	-----ENNP	72		
	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhh	eeee	t	eeee t		
	D VR D MG REDL D RHVD VH FE WGLP			GR		
Af	EDYVRVTLDMGLAREDLVADYARHVDGTVHLFERWGLPIWKT	FDQKYVKRGTVADNDSVRVATSSQSSQAGRMVEVGK	-----	168		
Dv	DDYVRVTLDMGLVREDLIFDLGRHVDSDVHLFEWGLPCWIKD	EGHENLDGAQAKAGSLRNGDDPVRSGRWQIMINGESYKCI	VAEAAK	179		
Cv	EDYVRVTLDMGLVREDLIFDLGRHVDSDVHLFEWGLPLMRN	PKTGAYQR	-----EGRWQIMIHGESYKCI	VAEAAK	146	
	hhhhheeeeeee	FFFFF	t	eeeeehhhhhhh	eee	
	LL	PN	AGAVGF R	F	A GGA	
Af	-----EEHLRESFSSSELLKGRNDFNAVAGAVGFSVREPKFYVFR	AKAVILATGGATLLFRPRSTGEAAGRTWYAFIDTGS	GYMGLKAGAMLT	257		
Dv	NALGEARIMERIFIVKLLDANTPNRVAGAVGFNLRAVEVHIFRS	NAMLVACGGAVNVYKPRSTGEGMGRWYVWVWAGSTYTMCAQVGA	EMT	272		
Cv	KS-----ADKVFNRICVTHLMDSEKPNRIAGAVGFNVRTGNY	HVFSKTVIVAAGGASNIYKPRSVGEAGRWYAFWSSGSAYGLL	IGAGAKMT	237		
	hhheeeeeee	t	eeeeee	thhhhhh	t	hhh
	E R	RFKDGYGPGVA FL K	N GE Y	L		
Af	QFEHRFIPFRKDGYPGVGAWFLFFKCKAKNAYGEEYIKTRAA	ELKYKPYGAAG	-----PIPTPLRNHQVMLEIMDGNQPIYMT	TERL	341	
Dv	MMENRFVPAFRKDGYPGVGAWFLFFKAKATNYKGEDYCATN	RMLKPYEDRGYAKGH	-----VIPTCLRNHMLREMRGRGPIYMDTKTA		358	
Cv	QMENRIVLARFKDGYPGVGAWFLFLKTYTQNLGEEYESKWW	POLQEMVGEYLD	PELSHRT	PIPTCLRNHALISEVNAGRGPIHMT	TEA	330
	hhh	hhhhhhhhhhhh	t	hhhhhhh	eeee	
	H	FL M V QA LWA	P	E	EPY GSH	G W GP
Af	LAE-LAGGDKKKLKHIIYEEAFEDFLDMTVSQALLWACONID	POEQPSEAPAEPIYIMSGSHSGEAGFWVCGPEDLMPEY	AKLFLPK	-----YNRM	430	
Dv	LQSTFANMTPEQKHLESEAWEDFLDMCVQANLWASMNIP	QPEERGSEIMPTPEYLLSGSHSGCCGIWVSGPDKWVPEDYK	VRASNGKI	-----YNRM	451	
Cv	FQD-----PHELEIGWENFLGMITVGOAVLWAAATDVP	KNENPELTSEPYVMGSHATCGGAWCSGPEVSPPEYFWG	-----YNRM	406		
	eeeeehhhhhhhh	t	hh	hhhhhhh	hh	hh
	TTV GL	D G H FS	K	Y P	T	VNP YI P
Af	TTVKGFLFAIGDCAGANPKFSRVRSLRQ	-----CSEGAVMFIPEQKNPE	-----IDDAVVEELKKAYAPMERFMQYKDLSTADDVN	PEYILPWQGLVR	521	
Dv	TTVEGLWTCADGVGASGHKFSGSHAEGRICGQMVWVWCL	DEKDYKPK	-----AIKESADELVKLIYRPYNYNMEGKAASDPVNP	PSYITPKNFM	543	
Cv	TTVEGLFGAGDAVGGTTPHAFSSGSFTGR	-----LAKAACKYIDDGKAEGRIVSDAQIERRQEIYKPM	EHYRVYRNEITAGSVNPNYINPROGLDR		499	
	hh	t	eeee	t	hh	
	L K DEY G Y T L	L ED KLAA	HEL R W E	HR T	H FR E R PG Y Y R D	D W
Af	LQKIMDEYAAGIATFYKTKMKLQRALELLAFKEDLEKLAARD	LEHLMRAWELVHRVVTAAEAHVREMLFRKETR	-----YNYRDTYDPELND	DEW	614	
Dv	LKCTDEYGGGVGTYYTTSAAALDTGFSLLGLEEDSLKLAARD	LEHLLRCWENYHRLMTVRLMHOIRFREESRY	-----YNYRDTYDPELND	DEW	636	
Cv	LQKIMDEYCGGVTVSYMTNENLNLGLKMKLEEDLEKLAARD	LEHLLRAWELKROLTSEAVLHETLFRKETR	-----YNYRDTYDPELND	DEW	592	
	hhhh	eeeeeeeeeeeeee				
	S D E K					
Af	KCFVCSKYDAEKDEWTFEKVPPYVQVIEWSF	644				
Dv	KCFVNSKYDPATGETKIFKAYYQIIFE	664				
Cv	EVLTVSRDPDTGEYTMKAPCYHLVDA	620				

Fig. 1. Alignment of deduced amino acid sequences from the structural genes for AprB (a) and AprA (b) subunits of APS reductases in *A. fulgidus* (Af), *D. vulgaris* (Dv) and *Chr. vinosum* (Cv). The alignment was performed with MACAW (Schuler et al., 1991) with the substitution matrix BLOSUM 62 (Henikoff & Henikoff, 1992). Blocks of statistically significant similarities among homologous polypeptides are boxed. Strictly conserved residues are given in the line immediately above the alignments. In the top line, the consensus secondary structures as calculated by SSPRED (Mehta et al., 1995; sspred@embl-heidelberg.de) and the SOPMA server (Geourjon & Deleage, 1995; deleage@ibcp.fr) are given: e, any sheet; E, amphiphilic sheet according to West & Hecht (1995); F, hydrophobic sheet; h, any helix; H, amphiphilic helix according to West & Hecht (1995); t, turn. Regions of statistically significant sequence similarity to succinate dehydrogenases, fumarate reductases and L-aspartate oxidases are indicated by double lines below the alignments. Degenerate primers for heterologous PCR were derived from the hexapeptides shown on a black background. Note that the composition of the figure reflects the arrangement of the structural genes (*aprBA*).

Fig. 2. Comparison of the results of Southern hybridization and heterologous PCR with enzymological evidence. (a) Detection of *apr* homologous regions in the genomes of representative sulfur-oxidizing bacteria by Southern hybridization with the heterologous probe Afwh5wh6^{DIG}. Cv, *Chr. vinosum* (*Xho*I/*Hind*III, 3.6 kb); Td, *T. denitrificans* (*Bam*HI, 1.4 kb and 4.5 kb); Cl, *Chl. limicola* f. sp. *thiosulfatophilum* (*Clal*, 4.8 kb); Em, *Ect. mobilis* (*Clal*); Ra, *R. adriaticum* (*Clal*); Rs, *R. sulfidophilum* (*Clal*); M, 1 kb ladder (Promega). Fragment sizes corresponding to the signals obtained are given in parentheses. Repeated experiments with different DNA loads consistently yielded the same results. (b) Amplification of *apr* homologous

(Lys-263), however, does not favour an ADP-binding fold (Wierenga *et al.*, 1986) and none of the programs used in this study predicted a suitable secondary structure. Recently, Moodie *et al.* (1996) stated that adenylate binding can be achieved by a large number of alternative arrangements. Adenylate-binding regions showed high frequencies of arginine, tyrosine and glycine, whereas glutamate rarely occurred. The region from residues 167 to 240, which is highly conserved in the AprA polypeptides and the related proteins succinate dehydrogenases, fumarate reductases and L-aspartate oxidases (Fig. 1b, double-underlined), is interrupted by a nonapeptide present only in the AprA sequences. In accordance with the observations of Moodie *et al.* (1996), this nonapeptide contains strictly conserved glycine, arginine, tryptophan and tyrosine residues (residues 212–220). It is, therefore, tempting to assume that both this nonapeptide and the strictly conserved undecapeptide mentioned above are involved in APS binding. This is supported by the observation that the undecapeptide in the AprA sequences is immediately followed by a stretch of 24 amino acid residues that does not occur in the aligned sequences of succinate dehydrogenases, fumarate reductases and L-aspartate oxidases. The latter sequences, however, contain a segment of almost the same size immediately before the undecapeptide, which has no counterpart in the AprA sequences (data not shown). This segment is known to comprise the active centre of succinate dehydrogenases and fumarate reductases (Schröder *et al.*, 1991).

While the AprA subunits of APS reductases from the chemotrophic sulfate reducers *A. fulgidus* and *D. vulgaris* and the phototrophic sulfur oxidizer *Chr. vinosum* do not differ fundamentally, there are obvious differences between the respective AprB subunits (Fig. 1a). As already stated by Speich *et al.* (1994), AprB from *A. fulgidus* contains an arrangement of cysteine residues characteristic for *Azotobacter*-type (7-Fe) ferredoxins; the same arrangement is found in AprB from *D. vulgaris*. In contrast, the cysteine arrangement in AprB from *Chr. vinosum* fits the pattern of *Clostridium*-type (8-Fe) ferredoxins (Iwasaki *et al.*, 1994), i.e. it lacks five amino acid residues in the amino-terminal cluster-binding region. Despite possessing an identical arrangement of

regions by heterologous PCR. (bi) Results of PCR with the primer pair wh53wh62; (bii) results of PCR with primer pair wh54wh62. DNA from the bacteria listed above and from *A. fulgidus* (Af) and *D. gigas* (Dg) served as templates. Note that the visible amplicons in lanes Em, Ra and Rs of panel (bii) were not of the anticipated size. Ethidium-bromide-stained gels and Kodak XAR-5 films were recorded with a CREAM CGS documentation system (Intas), sizes of restriction fragments and amplicons were determined using the CREAM 1-D software (Intas). (c) Verification of PCR results by Southern hybridization with the heterologous probe Afwh5wh6^{DIG}. The presence (+) or absence (–) of distinct signals obtained with each amplicon shown in (b) is qualitatively summarized. As the results for wh53wh62 and wh54wh62 amplicons were absolutely congruent, the statement is valid for either primer pair. (d) Enzymological evidence for the presence (+) or absence (–) of an APS reductase. References are given in the text.

cysteine residues, different spectroscopic properties were reported for the APS reductases from *A. fulgidus* and *D. vulgaris* (Lampreia *et al.*, 1991; Verhagen *et al.*, 1994). Therefore it would seem premature to discuss putative functional implications of the different cysteine arrangements before spectroscopic data for the APS reductase from *Chr. vinosum* are available. Whether the additional residues at positions 81, 82 and 93 in AprB from *Chr. vinosum* are of any functional or structural importance needs to be the subject of further studies.

The lack of signal peptides and transmembrane segments as predicted by SIGNALP (Rapacki, Center for Biological Sequence Analysis, Lyngby; <http://www.cbs.dtu.dk/signalp/cbssignalp.ms.html>) and PSORT (Nakai, Institute for Molecular and Cellular Biology, Osaka; <http://psort.nibb.ac.jp>) characterize the gene products AprA and AprB from *Chr. vinosum*, together with all other polypeptides examined in this study, as cytoplasmic and soluble. This result is in contradiction to the finding that APS reductase activity is firmly membrane-bound in *Chr. vinosum* (Schwenn & Biere, 1979). The situation may be analogous to the succinate-ubiquinone oxidoreductase from *E. coli*. Here, hydrophobic subunits anchor the hydrophilic flavo-protein and iron-sulfur protein subunits to the cytoplasmic membrane (Nakamura *et al.*, 1996).

Design of a heterologous DNA probe and PCR conditions for the detection and amplification of *apr* genes

A gene probe must meet the criterion of specificity and selectivity. AprA, the large subunit of the APS reductase from *A. fulgidus*, contains the putative substrate-binding site, region(s) that should be conserved in all APS reductases (Speich *et al.*, 1994). A DNA probe was prepared by PCR amplification and nonradioactive labelling of the entire *aprA* gene as specified in Methods.

Genomic DNA from representatives of the purple sulfur bacteria (*Chr. vinosum*, *Ect. mobilis*), nonsulfur purple bacteria (*R. sulfidophilum*, *R. adriaticum*), green sulfur bacteria (*Chl. limicola* f. sp. *thiosulfatophilum*), and thiobacilli (*T. denitrificans*) was subjected to Southern hybridization with the *aprA* gene as a probe. Fig. 2(a) shows the results of hybridization obtained under conditions of low stringency (55 °C). Distinct signals were only obtained with restricted DNA from *Chr. vinosum*, *T. denitrificans* and *Chl. limicola* f. sp. *thiosulfatophilum*.

The alignments of amino acid sequences of AprA and AprB revealed several highly conserved regions. For primer design, hexapeptides were chosen that were located as close as possible to the termini of the polypeptides to maximize amplicon size (Fig. 1). From the positions in the respective gene loci as given in Methods, wh53wh62 amplicons were expected to be in the range of 2.2 kb and wh54wh62 amplicons in the range of 1.6 kb. In addition to the bacteria listed above, DNA from *A. fulgidus* and *D. gigas* was used as template. Fig. 2(bi) and (bii) show the results of PCR

experiments with the primer pairs wh53wh62 and wh54wh62, respectively. While all other amplicons were obtained with high reproducibility under the conditions stated here, this did not apply for Tdwh53wh62 (Fig. 2bi). Obviously, the primer pair wh54wh62 worked less selectively than wh53wh62, as is shown by visible amplicons obtained with DNA from *Ect. mobilis*, *R. adriaticum* and *R. sulfidophilum* (Fig. 2bii). These amplicons, however, did not have the anticipated size (see above). To verify that the properly sized amplicons actually did and the improperly sized ones did not correspond to *apr* homologous regions, the amplicons were probed with Afwh5wh6^{DIG}. The qualitative results are summarized in Fig. 2c and were positive for both wh53wh62 and wh54wh62 amplicons.

All results correlate perfectly with enzymological data (Fig. 2d): whereas the presence of APS reductase in *Chr. vinosum*, *Chl. limicola* f. sp. *thiosulfatophilum*, *T. denitrificans* and *D. gigas* is well-documented (Dahl, 1996; Schwenn & Biere, 1979; Kirchhoff & Trüper, 1974; Bowen *et al.*, 1966; Lampreia *et al.*, 1990), the enzyme is generally considered to be absent in the *Rhodospirillaceae* and *Ectothiorhodospiraceae* (Neutzing *et al.*, 1985; Trüper & Fischer, 1982). Moreover, the amplification of *apr* homologous regions with the primer pair wh53wh62 proves the gene order *aprBA* in the genomes of *D. gigas*, *Chl. limicola* f. sp. *thiosulfatophilum* and *T. denitrificans*. The absolute consistency of all data demonstrates that the DNA probe and PCR conditions described in this work are excellent diagnostic tools and will aid in the acquisition of further data.

Description of the *dsr* locus from *Chr. vinosum* and alignment with sequences from *A. fulgidus* and *D. vulgaris*

A BLASTX analysis of a 2567 bp DNA region from *Chr. vinosum* led to the identification of two ORFs. Their inferred translation products exhibited high statistical similarities with smallest sum probabilities equal to or less than 9.2×10^{-75} to the subunits of the dissimilatory sulfite reductases from *A. fulgidus* and *D. vulgaris*. The first ORF (nt 22–1275) was found to encode a 417 aa polypeptide with a computed molecular mass of 46791 Da. Translation of the second ORF (nt 1330–2400) yielded a 356 aa polypeptide with a computed molecular mass of 39983 Da. Putative RBSs (AGGAG, GGAGG) are located immediately upstream of the two ORFs. The reported molecular masses of the two subunits of *Chr. vinosum* sulfite reductase, 42 kDa and 37 kDa (Schedel *et al.*, 1979), are in good agreement with the computed data for the gene products of ORF1 and ORF2, respectively. As Schedel *et al.* (1979) did not detect any other sirohaem-containing protein in *Chr. vinosum* and the sequence requirements for sirohaem-binding are met (see below), ORF1 and ORF2 very likely represent the structural genes *dsrA* and *dsrB* for the sulfite reductase described by Schedel *et al.* (1979). In contrast to the situation in *D. vulgaris* and *A. fulgidus* (Karkhoff-Schweizer *et al.*, 1995), an ORF homologous

Fig. 3. Alignment of deduced amino acid sequences from the structural genes for the α - (-A) and β - (-B) subunits of sulfite reductases from *A. fulgidus* (Af), *D. vulgaris* (Dv) and *Chr. vinosum* (Cv). The features are the same as for Fig. 1 with the exception that residues found in all sequences are given in the top line above the sequences. The positions of residues of the cys motif are shown on a black background.

to *dsr/dsvD* was not found downstream of *dsrB*. To avoid confusion, in the following text the sequences from *D. vulgaris* are also named *dsr* instead of *dsv* (Karkhoff-Schweizer *et al.*, 1995). The extent of similarity between the primary and secondary structures of the respective polypeptides from *A. fulgidus*, *D. vulgaris* and *Chr. vinosum* is depicted in Fig. 3. Again, the large number of residues conserved in all sequences is striking.

The most conserved sequence pattern in sirohaem-containing sulfite reductases and nitrite reductases is the succession of two cysteine clusters with the consensus sequences Cys-x₅-Cys and Cys-x₃-Cys. The existence of this 'cys motif' (Campbell & Kinghorn, 1990) is the essential prerequisite for coupling of the [Fe₄S₄]-sirohaem cofactor (Ostrowski *et al.*, 1989; Crane *et al.*, 1995). This means that the loss of only one of these cysteines would result in the inability to bind the cofactor. As in the dissimilatory sulfite reductases from *A. fulgidus* and *D. vulgaris* (Dahl *et al.*, 1993; Karkhoff-Schweizer *et al.*, 1995), only DsrA from *Chr. vinosum* contains a complete cys motif, whereas the cys motif in DsrB appears to be truncated (Fig. 3, positions shown on a black background). Another common feature of dissimilatory sulfite reductases is the existence of ferredoxin-like domains (Dahl *et al.*, 1993). In the polypeptides from *Chr. vinosum* these domains include residues 264–290 in DsrA and residues 211–240 in DsrB. Some differences are also evident between the polypeptides from the chemotrophic sulfate reducers and those from the phototrophic sulfur oxidizer. Both subunits of the enzymes from *A. fulgidus* and *D. vulgaris* contain histidine residues (His-44 in AfDsrA, His-186 in AfDsrB) and heptapeptides (residues 32–38 in AfDsrA, 112–118 in AfDsrB) that do not occur in the *Chr. vinosum* polypeptides, while Ser-270, Lys-330 and Lys-331 are only found in DsrA from *Chr. vinosum* (Fig. 3). These differences may reflect the role of sulfite reductase in the reductive versus the oxidative pathway.

The view that the structural genes encoding the α - and β -subunits of sulfite reductase resulted from a duplication of a common ancestral gene (Dahl *et al.*, 1993) is supported by several observations. (1) Segments of statistically significant similarity between the nucleotide sequences of *dsrA* and *dsrB* are still found with the Gibbs sampler (Lawrence *et al.*, 1993) implemented in MACAW. These segments correspond to amino acids 109–124 (67–82), 203–218 (160–175), 276–295 (226–245) and 360–376 (309–325) in CvDsrA (CvDsrB) and to the aligned sequences of the respective counterparts from *A. fulgidus* and *D. vulgaris* (Fig. 3). (2) There is a large extent of statistically significant similarity between the amino acid sequences of DsrA and DsrB (boxed regions extending over DsrA and DsrB sequences in Fig. 3) covering at least 70% of the total amino acid residues of each polypeptide. To support the assumption that the reading frame has always been similarly maintained in *dsr* sequences, multiple alignments with translation of all frames were performed (Lockhart *et al.*, 1996). This ruled out insertion and deletion events resulting in frameshifts.

Fig. 4. (a) Topologies of phylogenetic relationships of the subunits of APS reductases (Apr) and sulfite reductases (Dsr) from *A. fulgidus* (Af), *D. vulgaris* (Dv) and *Chr. vinosum* (Cv). The unrooted trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). Only unambiguously aligned residues as estimated by statistically significant sequence similarities were included for the analyses; gaps in the data sets were avoided. The distances from which the trees were inferred are based on the blocks of statistically significant similarities as delineated in Figs 1 and 3. The values according to the PAM 001 matrix (Dayhoff, 1978) for Af–Dv, Dv–Cv and Af–Cv, respectively, are: AprA, 0.87, 0.91, 0.87; AprB, 0.47, 1.05, 1.10; DsrA, 0.65, 0.98, 0.96; and DsrB, 0.58, 1.03, 1.05. The values represent the expected percentage change between two amino acid sequences. (b) Topology of the phylogenetic relationships of the α - and β -subunits of the dissimilatory sulfite reductases from *A. fulgidus*, *D. vulgaris* and *Chr. vinosum*. The distances from which the tree was inferred are based on the alignment shown in Fig. 3. The values are: AfDsrA–AfDsrB, 3.02; DvApsA–DvApsB, 2.98; and CvDsrA–CvDsrB, 2.70. The unrooted tree was calculated by the Fitch–Margoliash least-squares method (Fitch & Margoliash, 1967) without the assumption of a molecular clock. The sum of squares for the tree is 0.028, and the average per cent standard deviation is 3.173 with 2217 trees being examined. Bootstrap values (given in per cent of 500 replicates) that support particular nodes are indicated on the tree. The trees in (a) were constructed by the successive use of PROTDIST, NEIGHBOR and DRAWTREE. For the tree shown in (b) FITCH (with global rearrangements being allowed and jumbling of data set entry for 50 times) was applied instead of NEIGHBOR. Bootstrap analyses were performed by the successive use of SEQBOOT, PROTPARS and CONSENSE. All these programs are parts of the PHYLIP package (Felsenstein, 1993).

Phylogenetic analyses

The observed high long-range similarities in nucleotide (data not shown) and amino acid (Figs 1, 3) sequences, together with the similar physico-chemical properties of the polypeptides indicated that AprA, AprB, DsrA and DsrB from *Chr. vinosum* and from the sulfate reducers *A. fulgidus* and *D. vulgaris* did not evolve convergently but are true homologues (Doolittle, 1994). It therefore appeared justifiable to calculate evolutionary distances derived from the amino acid sequences.

The tree topologies in Fig. 4 show essentially no difference between the distances of the polypeptides

from *Chr. vinosum* to those of *D. vulgaris*, and of the polypeptides from *Chr. vinosum* to those of *A. fulgidus*, respectively. The distances from the *D. vulgaris* to the *A. fulgidus* polypeptides are equal (AprA) or smaller (all others) than those from the *Chr. vinosum* to the *A. fulgidus* polypeptides. The observed tree topologies are not congruent with the 16S-rRNA-based phylogeny of the source organisms which places the two proteobacteria *Chr. vinosum* and *D. vulgaris* far away from the archaeon *A. fulgidus* (Olsen *et al.*, 1994). Two different possibilities could explain the observed tree topologies. First, a recent lateral gene transfer could have occurred. However, lateral gene transfer is a rare event in prokaryotes (Cohan, 1996) and the fact that all *apr* and *dsr* genes from the proteobacteria *D. vulgaris* and *Chr. vinosum* show the same strong synonymous codon bias (data not shown), as would be expected for phylogenetically closely related organisms with similar G + C content (Osawa *et al.*, 1992) argues against lateral gene transfer. As the establishment of the optimized codon usage is regarded to be a very slow process (Diaz-Lazcoz *et al.*, 1995), the conspicuously different synonymous codon bias in the *apr* and *dsr* genes from *A. fulgidus* implies a primordial split of the *A. fulgidus* and the proteobacterial lineages. The existence of highly conserved stretches in interchanging sequence pairs (Figs 1, 3) is also incompatible with lateral gene transfer.

The observed tree topologies can be better explained by the assumption that the sulfur-metabolizing enzymes from sulfur-oxidizing and sulfate-reducing organisms are not orthologous but paralogous (Gogarten, 1994) proteins. In the latter case, the protogenetic (Benner *et al.*, 1989; Benner & Ellington, 1990) precursors of sulfite reductases and APS reductases would have evolved into two independent lineages with the enzymes working in the reductive direction in one and in the oxidative direction in the other prior to divergence into the imperia (Trüper, 1994) Bacteria and Archaea. A comparable concept has been suggested for the evolution of glutamate dehydrogenase gene families I and II (Benachenhou-Lahfa *et al.*, 1993). Our hypothesis of a dichotomic evolution of the sulfur-metabolizing enzymes is supported by the observation that monoclonal and polyclonal antibodies directed against APS reductase from *Desulfovibrio desulfuricans* strain G100A cross-react strongly with extracts from other sulfate-reducing bacteria, but only weakly if at all with extracts from the sulfur-oxidizing bacteria *T. denitrificans*, *Chr. vinosum* and *Chl. limicola* f. sp. *thiosulfatophilum* (Odom *et al.*, 1991). Polyclonal antibody against the sulfite reductase from *A. fulgidus* cross-reacts with the respective enzyme from the bacterial sulfate reducer *Thermodesulfobacterium commune* (Dahl *et al.*, 1993), but only very poorly with extracts from *Chr. vinosum* and *T. denitrificans* (data not shown). In addition, we were not able to amplify parts of the *dsr* loci from *Chr. vinosum* and *T. denitrificans* with essentially the same PCR primers that Karkhoff-Schweizer *et al.* (1995) used to amplify parts of the *dsr* loci from diverse bacterial sulfate reducers. Further-

more, Southern hybridizations with parts of the *dsr* loci from *A. fulgidus*, *D. gigas* (GenBank accession number U80961) and *Chr. vinosum* as probes revealed a distinctly higher similarity of the *dsr* locus from *T. denitrificans* to the *dsr* locus from *Chr. vinosum* than to the *dsr* loci from the two sulfate reducers (data not shown).

The tree shown in Fig. 4(b) strongly suggests that the duplication of the common ancestor of the *dsr* genes must have occurred prior to entering the lineages leading to the contemporary sequences, as α - and β -subunits are more closely related among themselves than to each other in one organism. The dyad symmetry of the tree can easily be explained when constant evolutionary rates of the α - and β -subunit lineages are assumed. This assumption was confirmed by the two-cluster test of Takezaki *et al.* (1995) as implemented in PHYLTEST (Kumar, 1996) with those sirohaem-binding proteins least distant to the α - and β -subunits as outgroup lineage (data not shown). The similar evolutionary distances between the α - and β -subunits from the three different organisms (3.02, 2.98 and 2.70 for *A. fulgidus*, *D. vulgaris* and *Chr. vinosum*, respectively; Fig. 4b) further suggest similar evolution rates for sulfite reductase in these organisms (Woese, 1987). Hence, the evolutionary distances between the subunits from *Chr. vinosum* and those from the two other prokaryotes are not overestimated by the differences in amino acid sequence (Iwabe *et al.*, 1989).

Conclusions

The phylogenetic analysis presented in this study allows general proposals about the evolution of dissimilatory sulfur metabolism to be made. The divergence into the oxidative and the reductive mode of dissimilatory sulfur metabolism was a protogenetic event. In the course of evolution, the set of enzymes optimized for chemotrophic sulfur oxidation was challenged in turn by the evolution of phototrophy (Nisbet *et al.*, 1995). Thus, dissimilatory sirohaem sulfite reductases and APS reductases in anoxygenic phototrophic bacteria predate the evolution of the photosynthetic apparatus. Following this view of early evolution, the single report of a dissimilatory sirohaem sulfite reductase in the *Chromatiaceae*, that is in *Chr. vinosum*, as well as the absence of APS reductases in the *Rhodospirillaceae*, *Ectothiorhodospiraceae* and some species of the *Chromatiaceae*, reflect the decreasing significance of these enzymes for the contemporary mode of anoxygenic phototrophy. The very recent observation that APS reductase is not an essential enzyme for photolitho-autotrophic growth of *Chr. vinosum* (Dahl, 1996) is consistent with this view. It is tempting to assume that this holds true for other anoxygenic phototrophic bacteria possessing an APS reductase (such as the *Chlorobiaceae*). Experiments analogous to those performed by Dahl (1996) will help to clarify the significance of APS reductase in other phototrophs. Sequencing of the structural genes for sirohaem sulfite reductases

and APS reductases, especially from chemotrophic sulfur oxidizers, will resolve whether this hypothesized scenario matches the actual course of evolution.

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