Characterization of the locus encoding the [Ni-Fe] sulfhydrogenase from the archaeon *Pyrococcus furiosus*: evidence for a relationship to bacterial sulfite reductases

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The *hydBGDA* genes, which encode the four subunits β , γ , δ and α of the [Ni-Fe] hydrogenase from the archaeon *Pyrococcus furiosus*, have been isolated and sequenced using a PCR/IPCR-based strategy. From the sequence analysis it appears that the four structural genes are tightly linked and organized in a single transcription unit. The *hydD* and *hydA* gene products are related to the small and the large subunits of several archaeal and eubacterial [Ni-Fe] hydrogenases with an overall degree of sequence relatedness ranging from 35% to 50% (identity + similarity). In particular, the amino acid sequence motifs involved in the accommodation of nickel and iron-sulfur clusters are conserved. In addition, the database search revealed that the *hydB* and *hydG* gene products are homologous to the *asrA*- and *asrB*-encoded subunits of the sulfite reductase enzyme from *Salmonella typhimurium*. This is particularly interesting in view of the recent finding that the *P. furiosus* hydrogenase appears to be a bifunctional enzyme endowed with both proton- and sulfur-reducing activities.

Keywords: hydrogenase, *Pyrococcus furiosus*, archaea, sulfur reduction, sulfite reductase

INTRODUCTION

Recently, largely through the efforts of Stetter and coworkers, a number of bacterial species able to grow well above 80 °C and belonging to the archaea have been isolated from several geothermic terrestrial and marine environments (Stetter, 1986).

Among these species the 'hyperthermophiles' grow optimally around 100 °C, are strict anaerobes, and most of them depend on the reduction of elemental sulfur (S^0) for growth. *Pyrodictium brockii* (Stetter *et al.*, 1983) and *Pyrobaculum islandicum* (Huber *et al.*, 1987) obtain energy for growth by S^0 respiration, the former being an obligate autotroph which reduces S^0 to H_2S with H_2 whereas the latter, a facultative heterotroph, reduces S^0 with either H_2 or organic substrates. On the other hand, *Pyrococcus furiosus* (Fiala & Stetter, 1986) can grow in the absence of

S^0 , fermenting simple and complex carbohydrates and producing H_2 and CO_2 as detectable products. In the presence of S^0 , H_2S is produced and the biomass yield is increased. The reduction of S^0 was postulated to take place to prevent the growth inhibitory effect of the H_2 produced during fermentation (Bryant & Adams, 1989). However, a recent study performed with an energy-limited continuous culture system has shown that in *P. furiosus* the reduction of S^0 is an energy-conserving reaction which directly or indirectly participates in the energy balance by an unknown mechanism (Schicho *et al.*, 1993).

In studying the sulfur metabolism of *P. furiosus*, Ma and coworkers found that elemental sulfur reduction is carried out by a sulfur reductase present in the cytoplasm (Ma *et al.*, 1993). Interestingly, the same authors showed that the sulfur reductase activity is associated with the previously characterized soluble hydrogenase, suggesting that the cytoplasmic production of S^{2-} might be coupled to the H^+ reduction by a bifunctional enzyme, a sulfhydrogenase.

P. furiosus hydrogenase, first isolated by Bryant & Adams (1989), is remarkably resistant to inactivation by tem-

Abbreviation: IPCR, inverse polymerase chain reaction.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence data reported in this paper is X75255.

perature and chemical agents. The enzyme remains 50% active after 21 h incubation at 80 °C and is not affected by the presence of SDS (0.1%) and β -mercaptoethanol (2%, w/v). Further characterization has shown that the 150 kDa enzyme is organized in an $\alpha\beta\gamma\delta$ tetrameric structure (Mura *et al.*, 1991).

To understand better the structural characteristics of the *P. furiosus* hydrogenase and its bifunctional properties associated with proton and sulfur reduction, we have isolated and characterized the DNA encoding this enzyme. In this communication we give the complete nucleotide sequence of the hydrogenase gene cluster. We have found that while two of the four subunits (α and δ) are homologous to the large and the small subunits of dimeric and multimeric [Ni-Fe] hydrogenases, the β and γ subunits share extensive homologies with the products of the sulfite reductase genes (*asrA* and *asrB*) from *Salmonella typhimurium* (Huang & Barrett, 1991). These findings and the genetic relationship of the *P. furiosus* *hyd* genes to the other [Ni-Fe] hydrogenases are discussed in light of the biochemical properties of the *P. furiosus* enzyme.

METHODS

Chemicals. Restriction and modification enzymes, purchased from Boehringer Mannheim or Promega, were used as recommended by the manufacturers. Reagents for DNA sequencing were supplied by US Biochemical and New England Biolabs. For the PCR and IPCR amplifications both *Taq* DNA polymerase (Boehringer Mannheim) and cloned *P. furiosus* DNA polymerase (Stratagene) were used.

Bacterial strains and growth conditions. *P. furiosus* (DSM 3638) was grown at 85 °C in closed static cultures using a synthetic seawater-based medium (SME) (Stetter *et al.*, 1983) supplemented with tryptone (5 g l⁻¹), yeast extract (1 g l⁻¹), L-cysteine (0.5 g l⁻¹) and 1 ml 0.2% resazurin solution as redox indicator. The pH was adjusted to 6.2 with HCl and anaerobic conditions were obtained by flushing the medium with argon for 30 min.

For large-scale growth, 15 l fermenters (Setric GI) were inoculated with 500 ml cultures grown in sealed bottles for 24 h. Fermentations were carried out at 95 °C with agitation (300 r.p.m.) in an argon atmosphere (300 ml min⁻¹). Growth was monitored by following the increase in turbidity at 660 nm and H₂ production was analysed using a Varian 3400 gas chromatograph. Cells were harvested after 24 h (final OD₆₆₀ = 0.25), washed in 50 mM Tris/HCl (pH 8) and stored at -80 °C. The biomass yield (dry weight) was 270 mg l⁻¹.

Escherichia coli strain TG1 [*supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB⁺ lacI^a lacZΔM15)*] was grown aerobically at 37 °C in Luria-Bertani (LB) medium. Competent *E. coli* TG1 cells were prepared and electroporated following the method previously described (Dower *et al.*, 1988). Recombinants were screened on LB agar plates containing ampicillin (100 µg ml⁻¹), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (40 µg ml⁻¹) and isopropyl β -D-thiogalactopyranoside (IPTG) (125 µg ml⁻¹).

Purification of *P. furiosus* hydrogenase. *P. furiosus* hydrogenase was partially purified as described by Bryant & Adams (1989). Sequencing grade enzyme preparations were obtained by gel elution. In particular, the partially purified enzyme was

run on a 10% (w/v) SDS-polyacrylamide gel and stained to visualize the hydrogenase activity (see below). The activity band was excised from the gel and electroeluted with a 422 Electroeluter (Bio-Rad) in Tris/glycine/0.1% SDS buffer (LeGendre & Matsudaira, 1989). The individual subunits of *P. furiosus* hydrogenase were completely dissociated by boiling the electroeluted enzyme (10 min at 100 °C) and by running it on a 10% SDS-polyacrylamide gel. Following the electrophoretic separation, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) as described elsewhere (Matsudaira, 1987). The N-terminal sequence of each subunit was determined by Edman degradation using a Beckman sequenator.

Proteins were determined spectrophotometrically with the Bio-Rad micromethod based on the Bradford assay (Bradford, 1976) using bovine serum albumin as the standard. SDS-PAGE was performed according to Laemmli (1970).

Hydrogenase activity. Hydrogenase activity was routinely determined by following H₂ evolution at 80 °C as described previously (Adams & Mortenson, 1984) using a Varian 3400 Gas Chromatograph equipped with a 5 Å molecular sieve column (1 m, 1/8 inch). One unit of hydrogenase activity is defined as the amount of enzyme which catalyses the production of 1 µmol H₂ min⁻¹.

Colorimetric visualization of hydrogenase activity on 10% SDS-polyacrylamide gels was performed at 70 °C as already described (Bryant & Adams, 1989).

Recombinant DNA techniques. The molecular biology methods were according to standard protocols (Sambrook *et al.*, 1989). When necessary, the subcloning of the PCR and IPCR (Ochman *et al.*, 1990) fragments was done using the pUC18 plasmid from Boehringer Mannheim or the pCRII TA cloning kit from Invitrogen.

Oligonucleotide synthesis. Oligodeoxynucleotides used for sequencing and amplifications were synthesized with Beckman DNA Synthesizers (models SM and OLIGO 1000). When required, the oligodeoxynucleotides were purified according to the specifications provided by Beckman. The following primers were used (from 5' to 3'; deg = degenerate): α 1deg, ATGAA-(AG)AA(TC)CT(TC)TA(TC)CT(TC)CC; α 2deg, TTTCC(TC)-TC(AT)AC(TC)CT(AT)GC(AT)AT; β 1deg, GA(AG)AA(TC)-AC(TA)TA(TC)GA(AG)TT(TC)CT(TC)GA; β 2deg, TT(AT)-AC(TA)GG(AT)GC(AG)TA(GA)AG(TC)TTTCC; δ 1deg, GGAAA(AG)GT(TA)AG(AG)AT(TA)GGATT(TC)TA; δ 2deg, AC(AT)AT(TC)TC(AT)GC(AG)TT(TA)TG(AT)AT; p1, GATAAGTTGTAATAACTCGTCCATCATAGCTAG; p2, CCAATCACCATTGATCATAT; p3, GAAAGACTTA-AAGACTGGGG; p4, GCGATAACCGTAGTTGATGAG; p5, GTCAGCTGAACAACTGTCCAG; p6, GCAGATAA-GTGCTTGCTTGTGG; p7, ATGGTTCAACTTCCTCT-ATTAC.

Isolation of hydrogenase genes. The forward and reverse primers were used to amplify either linear (PCR) or restriction-enzyme-digested and self-ligated (IPCR) *P. furiosus* chromosomal DNA. PCR and IPCR reactions (30 cycles of denaturation at 94 °C for 1 min, annealing at the appropriate temperature for 1 min and elongation at 72 °C for 2 min) were performed in 100 µl mixtures covered with 100 µl mineral oil. Reaction samples included: template genomic DNA (500 ng undigested DNA in the case of PCR, 20 ng restriction-enzyme-digested and self-ligated DNA in the case of IPCR), 200 µM dNTPs, 150 pmol degenerate primers or 100 pmol specific primers and 2.5 U of either *Taq* DNA polymerase or *P. furiosus* DNA

Fig. 1. (a) Restriction map and physical organization of the 4180 bp fragment carrying the *P. furiosus* *hydBGDA* genes encoding the β , γ , δ and α hydrogenase subunits. The thick arrows indicate the coding regions and the orientation of the four genes. (b) Schematic representation of the PCR and IPCR fragments used to isolate the entire hydrogenase gene cluster. Arrowed lines indicate the amplification directions.

polymerase. The amplification reactions were carried out using a Perkin Elmer Cetus thermal cycler.

Nucleotide sequencing and analysis. DNA sequencing was performed on both strands by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 (US Biochemical) and the CircumVent (New England Biolabs) sequencing kits. Routinely, different plasmids carrying the same IPCR fragment and different IPCR fragments covering the same DNA region were sequenced to avoid sequencing errors due to the non-perfect fidelity of polymerases.

The strategy used for the isolation and sequencing of the hydrogenase cluster is given in Fig. 1. Initially, using the $\delta 1$ deg/ $\alpha 2$ deg primer combination a fragment of about 700 bp was obtained. This suggested that the α subunit is linked to and preceded by the δ subunit coding sequence in the hydrogenase cluster. The 700 bp fragment containing the δ subunit gene and the 5' region of the α subunit was sequenced. Subsequently, the genomic DNA was digested with different restriction enzymes, self-ligated and amplified with two divergently oriented oligonucleotides (p1/p2) annealing to the 5' ends of the δ subunit gene and the α subunit gene, respectively. The analysis of these IPCR products allowed us to complete most of the sequence of the γ subunit and to extend further the sequence of the α subunit. The remaining part of the γ subunit and most of the β subunit were isolated by PCR performed with the convergently oriented primers p3 and p4 corresponding to the 5' ends of the β and γ subunits, respectively. The entire β subunit and the putative promoter region were obtained by amplifying the *Bam*HI-cut, self-ligated chromosomal DNA with two divergently oriented primers (p6/p7) annealing to the internal part of the β subunit. Finally, the entire α subunit was amplified from the *Eco*RI-cut, self-ligated genomic DNA using a combination of primers (p5/p2) corresponding to the 5' ends of the α and γ subunit genes, respectively.

The sequence was compiled and analysed by using the PC/GENE software package (IntelliGenetics). Multiple amino acid sequence alignments were performed using the MACAW program (Schuler *et al.*, 1991).

RESULTS AND DISCUSSION

Isolation and sequencing of the *P. furiosus* hydrogenase (*hyd*) genes

The four hydrogenase subunits having molecular masses of 50 000, 43 000, 33 000 and 31 000 Da were purified from a SDS-polyacrylamide gel as described in Methods and N-terminal sequenced. Degenerate forward and reverse primers were designed on the basis of the N-terminal sequences of the α , β and δ subunits and were used in initial PCR amplifications of the *P. furiosus* genomic DNA. The degeneracy of the oligonucleotides was lowered by taking into account the codon usage in the glyceraldehyde-3-phosphate dehydrogenase gene of the phylogenetically related organism *Pyrococcus wosei* (Zwickl *et al.*, 1990). The amplification of the 5' end of the γ subunit DNA was not attempted because of the limited length of the amino acid sequence available. The DNA sequences of the α , β and δ PCR products were in perfect agreement with the N-terminal sequences of the three subunits.

The knowledge of the sequences of the 5' ends of the *hydA*, *hydB* and *hydD* genes allowed us to determine the nucleotide sequence of the entire region containing the four genes using a strategy, described in Methods, based on the analysis of different PCR and IPCR products. The restriction map and the physical organization of the 4180 bp fragment carrying the *P. furiosus* *hyd* genes is shown in Fig. 1.

Sequence analysis

The nucleotide sequence and the derived amino acid sequences of the DNA region comprising the *P. furiosus* *hyd* genes are shown in Fig. 2.

Fig. 2. For legend see facing page.

(a)		
<i>P.f.</i>	13	SCYGCQLQLAMMDELLQ
<i>M.t.</i>	14	GCSGCHLSIADFHGKII
<i>M.v.</i>	11	GCTGCLISLTDTYEKLL
<i>M.v.</i>	11	CCSGCHISLIDLHEDLL
<i>D.f.</i>	17	ECTGCTEAAIRTIKPYI
<i>D.g.</i>	16	ECTGCSESLLRVTDPYV
<i>R.c.</i>	16	ECTCCSESFIRSAHPLA
<i>B.j.</i>	16	ECTCCSESFIRSAHPLV
<i>A.v.</i>	16	ECTCCSESFIRSAHPLV
<i>E.c.</i>	16	ECTCTESFIRSAHPLA
		* *
(b)		
<i>P.f.</i>	43	RFFEAITIGKKLEEALAIYPRICSFCSAAHKLTALEAAEKAVGFVPREEIQA
<i>M.t.</i>	39	RGFEKFLQGRPIEEAPRIVPRICGICDVQHLLAAKAVDACFGFEPEDVLP
<i>M.v.</i>	41	RGFEKFLVGKPAEFAPIAVSRCFCGICPIAHATSAVEAIEDACGIVPPKDGLL
<i>M.v.</i>	22	RGFEKFLGRIEDAPIFTPRICGICQVSHHLASAKAVDNVFGVKIPETANM
<i>D.f.</i>	50	RGLEIILKGRDPRDAQHFTQACGVCTYVHALASSRCVDDAVKVSIPANARM
<i>D.g.</i>	43	RGLEMILKGRDPRDAQHFTQACGVCTYVHALASVRAVDNVCVGKIPENATL
<i>R.c.</i>	53	RGLEVILKGRDPRDAWAFTERICGVCTGTHALTSVRAVESALGITIPDNANS
<i>B.j.</i>	53	RGIEVILKNRDPDAWAFTERICGVCTGTHALTSVRAVENALGITIPENANS
<i>A.v.</i>	52	RGLEVILKGRDPRDAWAFVERICGVCTGTHALTSVRAVEDALDIRIPYNAHL
<i>E.c.</i>	54	RGLEIILQGRDPRDARAFVERICGVCTGVHALASVYAIEDAIGIKVPDNANI
		* * * * *
	409	EMVVRAYDPCISCSVH
	433	EMVIRAYDPCLSATH
	377	EHVVRAYDPCVSCATQ
	419	EMVIRAYDPCLSATH
	533	LRTVHAFDPCIACGVH
	521	LRTVHSYDPCIACGVH
	567	LRTLHSFDPCLACSTH
	566	LRTIHSFDPCLACSTH
	572	LRTLHSFDPCLACSTH
	567	LRTLHSFDPCLACSTH
		*** *

Fig. 3. Comparison of the conserved regions of the small (a) and the large (b) subunits of several [Ni-Fe] hydrogenases. (a) Regions comprising the putative 2Fe- or 4Fe-type clusters at the N- and C-termini of the small subunits. (b) Regions comprising the nickel-coordination sites at the N- and C-termini of the large subunits. *P.f.*, *P. furiosus*; *M.t.*, *M. thermoautotrophicum* (small and large subunits of the *mvh* operon); *M.v.*, *Methanococcus voltae* (small and large subunits of the *frc* and *vhc* operons); *D.f.*, *Desulfovibrio fructosovorans*; *D.g.*, *Desulfovibrio gigas*; *R.c.*, *Rhodobacter capsulatus*; *B.j.*, *Bradyrhizobium japonicum*; *A.v.*, *Azotobacter vinelandii*; *E.c.*, *E. coli* (small and large subunit of the hydrogenase-1 operon). Identical residues are marked with asterisks. The numbers indicate the positions of each protein sequence within the corresponding subunit.

The genes corresponding to the α (*hydA*), β (*hydB*), γ (*hydG*) and δ (*hydD*) subunits encode polypeptides of 428, 367, 292 and 260 amino acids, respectively. The calculated molecular masses of the subunits (48 700, 41 800, 33 200 and 29 600 Da, respectively) are in agreement with those estimated by SDS-PAGE analysis.

The *hydG*, *hydD* and *hydA* genes begin with ATG codons, whereas *hydB* starts with the rare initiation codon GTG. GTG and TTG have already been found as initiator codons in other archaeal genes (Alex *et al.*, 1990; Halboth & Klein, 1992; Zwickl *et al.*, 1990).

The comparison of the N-terminal protein sequences with the corresponding deduced amino acid sequences indicates that none of the subunits are post-translationally processed at the amino-terminus, with the exception of the δ subunit, which lacks the initial methionine. There-

fore the enzyme, in agreement with the experimental data, appears to be a cytoplasmic protein.

The *hydBGDA* genes are closely linked: the stop and the start codons of the *hydB/hydG* genes and *hydD/hydA* genes are overlapping, whereas *hydG* and *hydD* are separated by 10 nucleotides. Each start codon is preceded by the typical ribosome-binding site found in most of the methanogen genes sequenced so far (Brown *et al.*, 1989). Finally, two adjacent archaeal transcription termination signals (Brown *et al.*, 1989) are located immediately downstream from the *hydA* stop codon.

On the basis of the overall organization of the *P. furiosus* *hyd* genes it appears that, like other multimeric hydrogenases (Przybyla *et al.*, 1992; Reeve & Beckler, 1990), the enzyme is encoded by a single transcription unit.

As far as the nucleotide composition is concerned, the

Fig. 2. Nucleotide sequence of the locus encoding the [Ni-Fe] hydrogenase of *P. furiosus*. The amino acid sequence of each open reading frame is given in one-letter code under the DNA sequence. Indicated are: the promoter region (box B), the putative ribosome-binding sites (asterisks), the start codons of the four subunits (short arrows), the transcription initiation site (vertical arrow next to box B) and the transcription termination signals (dotted lines). Amino acid residues determined by N-terminal sequencing are underlined. The initiator methionine of *hydD* is in parentheses since it is absent in the protein sequence.

G + C content of the four structural genes (44.3%) is higher than the average value calculated by Fiala & Stetter (1986) for the total genome of *P. furiosus* (37.1%).

The most striking observation emerging from the analysis of the codon usage is the marked preference for AGG and AGA among the arginine codons. This is a feature in common with many other archaeal genes (Brown *et al.*, 1989; Tiboni *et al.*, 1993; Uemori *et al.*, 1993; Zwickl *et al.*, 1990).

Experimental data indicate that the structural elements of the constitutive archaeal promoters can be recognized in the T/CTTAT/AA ('box A') and in the ATGC ('box B') sequences. Box B, located 29–24 nucleotides downstream from box A, generally represents the site where transcription initiation takes place, GTP being the preferred initiator nucleotide. Box A appears to play the most important role in promoter recognition and transcription initiation, as shown by the sequence analysis of the archaeal promoters so far characterized (Hain *et al.*, 1992) and by *in vitro* studies carried out with both total protein extracts and partially purified RNA polymerases (Hain *et al.*, 1992; Hausner *et al.*, 1991).

When the *hyd* operon was scanned for promoter sequences, a box B motif was localized just upstream from *hydB* (underlined in Fig. 2). However, in the same region, the box A motif is absent if the AAATTT sequence (centred 25 bases upstream from box B) and the TAA sequence (centred 32 bases upstream from box B) are not considered significant.

Comparison of *hydBGDA* gene products

1. *hydD* and *hydA* genes. The [Ni-Fe] hydrogenases have been classified on the basis of the number of subunits they are composed of. Although the presence of the gene for the large subunit is the sole feature common to all known nickel-containing enzymes, all the heterodimeric (two subunits) and many multimeric hydrogenases contain small subunits sharing a variable number of conserved cysteines, the function of which is to bind iron-sulfur clusters (for reviews see Przybyla *et al.*, 1992; Reeve & Beckler, 1990; Wu & Mandrand, 1993).

Comparison of the *hydBGDA* gene products with the [Ni-Fe] hydrogenases from different bacteria shows that the *hydD* and *hydA* products are clearly related to the small and large hydrogenase subunits, respectively (Figs 3 and 4).

The *P. furiosus* small subunit shows the highest degree of sequence identity with the *Methanococcus voltae* small subunits (27.6–36.4%) (Halboth & Klein, 1992), the *Methanobacterium thermoautotrophicum* MvhG protein (31.8%) (Reeve *et al.*, 1989) and with two eubacterial sequences, the *Acetomicrobium flavidum* HydB (35.4%) (G. M. Mura and others, unpublished) and *Alcaligenes eutrophus* HoxY proteins (29.5%) (Tran-Betcke *et al.*, 1990). Interestingly, the homology with the last two subunits is relatively high although the C-termini of both HoxY and *A. flavidum* HydB appear to be truncated by

approximately 50 amino acids. The homology with other small subunits from eubacterial origin is slightly less pronounced (22.2–27.2% identity).

The small subunit of the *P. furiosus* hydrogenase contains 13 cysteines out of a total of 261 amino acids (4.9%); nine of them are located at conserved positions when compared to other hydrogenase small subunits and of these, four are arranged in the C-X-X-C pattern at the N- and C-termini (Fig. 3a).

The large subunits of both archaeal and eubacterial [Ni-Fe] hydrogenases share several regions of well-conserved amino acid sequence with the *P. furiosus* α polypeptide (HydA). In fact the overall sequence identity between HydA and the corresponding archaeal sequences ranges from 29.2% to 34% whereas the identity with the eubacterial sequences goes from 27.8% to 32%. Within two of the most highly conserved regions, four cysteinyl residues participate in the R-X-C-X-X-C-X-X-H motif at the N-terminus and in the D-P-C-X-X-C-X-X-H motif at the C-terminus (Fig. 3b). This pattern, present in all the nickel-containing hydrogenases sequenced so far, was proposed to represent the nickel hexacoordination site of the enzyme. Indeed, Przybyla and coworkers have recently shown by site-directed mutagenesis that the arginine and the cysteine of the N-terminal box and the aspartic acid, the proline and the cysteine of the C-terminal box are required for the activity of the [Ni-Fe] hydrogenase-1 in *E. coli* (Przybyla *et al.*, 1992).

As far as the relative position of the *hydA* and *hydD* genes within the gene cluster is concerned, the gene encoding the hydrogenase small subunit (*hydD*) precedes the large-subunit-coding sequence (*hydA*). This organization is typical for all the [Ni-Fe] deazaflavine reducing hydrogenases sequenced so far and is conserved even in those cases where, in addition to the small and large subunits, other genes are found in the hydrogenase cluster (Fig. 4).

In conclusion, the α and δ subunits of the *P. furiosus* hydrogenase show the highest degree of homology with the large and the small subunits of hydrogenases from other archaea. Therefore the enzyme can be grouped with the class IV hydrogenases according to the recent classification by Wu & Mandrand (1993). The similarity with the enzymes of class II, which includes the hydrogenases from *Desulfovibrio* species, is less pronounced, although the relative positions of the cysteine residues of the *P. furiosus* small subunit and the class II subunits appear to be generally conserved. Since it has recently been shown by crystallographic analysis that 10 of the cysteine residues of the *Desulfovibrio gigas* small subunit provide the coordination sites for one [3Fe-4S] cluster and two [4Fe-4S] clusters (one [4Fe-4S] cluster in the N-terminal domain and the remaining clusters in the C-terminal domain) (Volbeda *et al.*, 1994), in the *P. furiosus* hydrogenase the mechanism of electron transfer for H₂ production might be similar to that of the *D. gigas* enzyme.

On the other hand, the homology between HydD and HoxY of *A. eutrophus* appears to be relatively high (the *A. eutrophus* hydrogenase has also been included in class IV

Fig. 4. Schematic representation of the gene organization found in a few dimeric and multimeric [Ni-Fe] hydrogenase clusters.

by Wu & Mandrand, 1993) but, since HoxY has a single [4Fe-4S] cluster (Przybyla *et al.*, 1992) and lacks the C-terminal domain, the electron transfer system of the two enzymes might be different.

2. *hydB* and *hydG* genes. The homology search carried out with the *hydB* and *hydG* gene products identified a significant degree of sequence identity with the AsrA and AsrB subunits of the *S. typhimurium* sulfite reductase (31.7% and 34.6%, respectively) (Huang & Barrett, 1991) (Fig. 5).

Salmonella is unique among *Enterobacteriaceae* because it is endowed with two sulfite reductase systems. The first one, common to many *Enterobacteriaceae* and coded by the genes *cysJ* and *cysI*, is associated with the cysteine biosynthetic pathway and is responsible for the H₂S-dependent conversion of *o*-acetylserine into cysteine. This sulfite reductase is a complex NADPH-dependent $\alpha_8\beta_4$ enzyme which contains FAD, [4Fe-4S] clusters and sirohaem to carry out the six-electron reduction of sulfite to sulfide without release of intermediates.

The second sulfite reductase system confers on *Salmonella* the unique ability to produce significant quantities of H₂S from sulfite under anaerobic conditions. From the recent work of Huang & Barrett (1991) it has been shown that this sulfite reduction system can be ascribed to a metabolic pathway which is (i) independent from the cysteine biosynthetic pathway, (ii) activated under strict anaerobic conditions, (iii) linked to NADH rather than NADPH

oxidation and (iv) regulated by available electron acceptors rather than by cysteine.

Three proteins are required for anaerobic H₂S evolution, the products of the genes *asrA*, *B* and *C*. These proteins contain the structural elements which are necessary for sulfite reduction and are conserved in the sulfite reductase enzyme of the cysteine biosynthetic pathway. In particular, AsrA and AsrC harbour typical ferredoxin-like [4Fe-4S] clusters (C-X-X-C-X-X-C-X-X-X-C-P), AsrC carries the sirohaem-binding sequences and AsrB contains two types of sequences that resemble the flavin-binding domains of a number of reductases (Huang & Barrett, 1991) and regions found in many nucleotide-binding enzymes, respectively.

The similarities between AsrA and HydB and AsrB and HydG appear to be evenly distributed along the sequences apart from a relatively poor sequence homology between the N-terminal regions of AsrB and HydG. HydB appears to contain the ferredoxin-like cluster and HydG the nucleotide and the FAD-binding domains. This latter observation is particularly interesting in the light of very recent work (Smith *et al.*, 1994) which demonstrates that NADPH rather than ferredoxin is the physiological electron carrier of the *P. furiosus* hydrogenase.

The similarities between HydB and AsrA and between HydG and AsrB are also particularly interesting considering that Ma *et al.* (1993) have recently demonstrated that the *P. furiosus* hydrogenase can catalyse polysulfide

(a)	
HydB	<u>MRYVKLPKENTYEF</u> L-ERL-KDWGKLYAP-VKI-SDKFYD-----FREIDDVRKIEFHYNRTIMPPKKFFFKPREKLFEDISKPEYREV 81
AsrA	MA- <u>IKITPDE</u> -FSLLIQRLNKKW-RVPAPSAEFRGGRFSDTDNIIYQRI SGWRDLIWE-EKSEMSPTIIAPITETLIFYFDKDTIQIAET 86
HydB	<u>IEEVEPFIIFGVHACDIYGLKILDTVYL-DEFPDKY-YKVRREKGIIGISCMPDEY--CFCNLRETDFAADDGFDLF--FHELPGDWLVR</u> 165
AsrA	-- <u>DTSPIIIFA-RACDINAMSRLDYMYLSNGNNSDYSYQLLREHIRFVLIECE-ESFENCFCVSMGTN-KTDCYSAAMRFS</u> --EGALVS 169
HydB	<u>VGTPTGHR</u> LVDKNIKLFEEVTDKIDICAF-RDF-EKRRQAFQ-----YHEDWGNLRYLLELEMEHPMWDEEADKCLACGICNTTCPTCRCY 249
AsrA	IRDP---- <u>FIEAAIQGLGGEAD</u> -----YTPSFVSENRETVTVPDSVCHDP-QKIRDIL---THEPLWDAYDRCISCGRCTTGCPTCTCY 246
HydB	<u>EVQDIV-NLDGVTGYRERRRWDSCQFRSEGLVAGGHNFRPTKDRFNNRYLCK-NAYNEKLGLSY-CVCGCRCTAFCPANISFVGNLRRIL</u> 336
AsrA	<u>SVFDVAYDENPQGERRRQWASCMVPGFSOMAGGEGFREKPGERLRYALHKVNDYKARNGIEHMCVCGCRCDRCQYIKF--SL--II</u> 332
HydB	<u>GLEENKCPPTVSEEI</u> PKRGFAYSSNIRGGV 367
AsrA	---- <u>NKMTAAVRQAL</u> -----AEEA 347
(b)	
HydG	<u>MMLP--KEIMMPN--DNPYALERVKVLKVYSLTETEKLF</u> FRFEDPELAEKWTFKPGQFVQLTIPGVGEVPISICSSPMRKGFPELCIRKA 87
AsrB	<u>MSHCSCDKPQHSLLAAAY</u> -----RILSITRHTPLE--WNFRVA-VDFFAHW---GQFVEVSLPRVGEAPISVSDYG--DGWIDLLIRNV 77
HydG	<u>GRVTTVVERLKPQD</u> TVLVRGPIYNGFFVDE-WEGMDLLIIAAGLGTAPLRSVFLYAMDNWKYGNITFINTARY-GKDLIFYKELEAMKDL 176
AsrB	<u>GKVT</u> SALFTLKEGDNVWLRCGYNGYPVDTLREKP--LLVVAGGTGVAPVKGMLRYFVENPQEIGQLDMILG--YKNRDCVLYK--EEMATW 163
HydG	<u>AEAENVKIIQSVTRDPNWPGLKGRPQQFIVEANTNPKNTA</u> VAIC-GPPFRMYKSVFEALINYGYRPENIFVTLERRMKCGIGKCGHCNVGTS 266
AsrB	<u>RGKENLVLT</u> DEGEADRYQI-GRVTDRLADMTLSIDTMDQAIVVGGPPIMITFTVKMLLQKGLKPEQIWVDYERPMACSVGKCGHCRNGEV 253
HydG	<u>TSWKYICKDGPVFTYFDIVSTPGLLD</u> 292
AsrB	---- <u>YVCTDGP</u> IPNY--AVAQR-FAD 272

Fig. 5. Comparison of the *P. furiosus* *hydB* and the *hydG* gene products with the *asrA* and *asrB* gene products of the sulfite reductase enzyme from *S. typhimurium*. Single and double points represent amino acids similarity and identity, respectively. In (a), the highly conserved [4Fe-4S] ferredoxin-like sequences are underlined. In (b), the conserved cysteines within the C-X-X-C motif of the 2Fe- or 4Fe-type cluster are marked by asterisks and the sequences found in many nucleotide-binding enzymes are underlined.

reduction to H_2S , allowing the disposal of excess reductant using either protons or polysulfides as electron acceptors.

Although the reduction reactions from sulfite to sulfide and from sulfur to sulfide are obviously chemically different, it is tempting to speculate on the existence of a common ancestor which divergently evolved to give two enzymes specialized in the reduction of sulfur at different oxidation states. The absence in the *P. furiosus* enzyme of sequences similar to AsrC, the subunit containing the sirohaem-binding domain which appears to be indispensable for sulfite reduction and conserved in both

Salmonella sulfite reductases, suggests a key role for AsrC in the six-electron reduction of sulfite to sulfide.

The homology studies of the *P. furiosus* hydrogenase described above suggest that the β and γ subunits might be involved in sulfur metabolism whereas the hydrogenase activity could be associated with the δ and α subunits. It would be interesting to test whether the HydB-HydG and the HydA-HydD dimers could be functionally dissociated from each other by testing the sulfite reductase activity of HydB-HydG and the hydrogenase activity of HydA-HydD. The organization of the hydrogenases in complex enzyme structures endowed with more than one

activity is not unusual. Typical examples are the four-subunit *A. entrophus* hydrogenase in which the diaphorase activity can be separated from the hydrogenase activity (Tran-Betcke *et al.*, 1990) and the H₂:heterodisulfide oxidoreductase complex of *M. thermoautotrophicum* in which the heterodisulfide-reductase activity can be separated from the F₄₂₀-non-reducing hydrogenase (Setzke *et al.*, 1994). Both genetic analysis such as heterologous gene expression and biochemical studies aimed at reconstituting the hydrogenase from its purified subunits might help in elucidating this interesting structural and functional aspect of the *P. furiosus* hydrogenase.

Ma *et al.* (1993) have shown that all the hydrogenases they tested show a significant sulfur reductase activity, regardless of the number of subunits they are composed of and besides the fact that none of the hydrogenases except the *P. furiosus* enzyme share homology to the *asr* genes.

This can be reconciled with our hypothesis that the structural elements required for sulfur reduction, which in the *P. furiosus* hydrogenase are embedded in the β and/or γ subunit(s), can either be localized on subunit(s) dispensable for the hydrogenase activity or inserted into the hydrogenase subunit(s). A careful analysis of many hydrogenase sequences suggests that the ferredoxin-like motifs, which are present on HydB and conserved in the sulfite reductase systems, might be involved in sulfur reduction. In fact they are also found in a large number of hydrogenases. For example, ferredoxin-like patterns are found in the large subunit of the Fe-only hydrogenase of *Desulfovibrio vulgaris* (Stokkermans *et al.*, 1989; Voordouw & Brenner, 1985), in the single-subunit Fe-only hydrogenase of *Clostridium pasteurianum* (Meyer & Gagnon, 1991), in the HybA and HycB subunits of the multimeric [Ni-Fe] hydrogenases-2 and -3 of *E. coli* (Przybyla *et al.*, 1992; Böhm *et al.*, 1990), in the MvhB and FhrG subunits of the *M. thermoautotrophicum* hydrogenases (Reeve *et al.*, 1989; Alex *et al.*, 1990; Bokranz *et al.*, 1988), in the VhcB subunit of *Methanococcus voltae* VHU hydrogenase (Halboth & Klein, 1992) and in the HoxU subunit of the *A. entrophus* NAD⁺ hydrogenase (Tran-Betcke *et al.*, 1990).

As already suggested by Ma *et al.* (1993), one could speculate on the presence, in the ancestor hydrogenase, of a high sulfur reductase activity to cope with and take advantage of the high-sulfur-containing environment. During the sulphydrogenase evolution nature has taken the option of either maintaining the structural elements required for sulfur reduction on independent subunits (as it is the case for the *P. furiosus* hydrogenase) or concentrating them on the same subunit(s) used for the proton reduction/H₂ oxidation activity. The former situation should provide the enzyme with a more specialized sulfur reduction activity, the latter with a predominant hydrogenase activity.

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