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Review

Heterodisulfide reductase from methanogenic archaea: a new catalytic role for an iron-sulfur cluster

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Abstract

Heterodisulfide reductase (HDR) from methanogenic archaea is an iron-sulfur protein that catalyzes reversible reduction of the heterodisulfide (CoM-S-S-CoB) of the methanogenic thiol-coenzymes, coenzyme M (CoM-SH) and coenzyme B (CoB-SH). Via the characterization of a paramagnetic reaction intermediate generated upon oxidation of the enzyme in the presence of coenzyme M, the enzyme was shown to contain a [4Fe-4S] cluster in its active site that catalyzes reduction of the disulfide substrate in two one-electron reduction steps. The formal thiyl radical generated by the initial one-electron reduction of the disulfide is stabilized via reduction and coordination of the resultant thiol to the [4Fe-4S] cluster.

Keywords: disulfide reductase; ferredoxin:thioredoxin reductase; heterodisulfide reductase; iron-sulfur proteins; methanogenic archaea.

Introduction

Iron-sulfur clusters in biological systems participate in a broad range of functions, including sensing and regulation, redox and non-redox catalysis, structural roles and electron transfer (Beinert, 2000). In recent years, a new function for iron-clusters has been identified in a number of enzymes that utilize a [4Fe-4S] and *S*-adenosylmethionine (AdoMet) to generate catalytically essential radicals (Fontecave et al., 2001). In other enzymes that catalyze radical reactions, such as benzoyl-CoA reductase, iron-sulfur clusters have also been shown to possess essential functions (Boll et al., 2001).

An additional new role for iron-sulfur clusters has emerged in recent years, as two disulfide reductases, heterodisulfide reductase (HDR) and ferredoxin:thioredoxin reductase (FTR), were found to utilize a [4Fe-4S] cluster to mediate the reductive cleavage of a disulfide substrate in two one-electron reduction steps (for a recent review see Walters and Johnson, 2004). Data obtained with both enzymes strongly indicate that the role of the [4Fe-4S] cluster is to mediate electron transfer to the substrate disulfide and to stabilize the formal thiyl radical that results from initial one-electron reduction of the disulfide via reduction and coordination of one thiol to a site on the resultant oxidized $[4Fe\text{-}4S]^{3+}$ cluster. HDR and FTR represent evolutionarily distinct enzymes. They are not sequence-related and share no sequence similarity with enzymes belonging to the well-characterized family of pyridinenucleotide disulfide oxidoreductases. These latter enzymes catalyze the electron transfer between NAD(P)(H) and a disulfide/thiol (Williams, 1995; Williams et al., 2000). Thioredoxin reductase, glutathione reductase and lipoamide dehydrogenase are prominent members of this enzyme family. These enzymes are homodimeric flavoenzymes, having a redox-active disulfide and a FAD in each monomer. The flow of electrons is from NAD(P)H to the FAD and from reduced FAD to the active-site disulfide, and finally from the active-site dithiol to the disulfide substrate. This cascade only involves two-electron transfer reactions.

In this review we summarize our current knowledge on heterodisulfide reductase with a special focus on characterization of the active-site iron-sulfur cluster. The enzyme is compared with the non-sequence-related ferredoxin:thioredoxin reductase, which also uses an active-site iron-sulfur cluster to mediate the reduction of a disulfide substrate.

Physiological function of heterodisulfide reductase and basic biochemical properties of the enzyme

Heterodisulfide reductase plays a key role in the energy metabolism of methanogenic archaea. Methane formation in these organisms is coupled to the formation of the heterodisulfide (CoM-S-S-CoB) of coenzyme M (CoM-SH) and coenzyme B (CoB-SH) in a reaction catalyzed by methyl-coenzyme M reductase (Thauer, 1998). Heterodisulfide functions as the terminal electron acceptor of an energy-conserving electron transport chain (Hedderich et al., 1998; Deppenmeier et al., 1999). The reducing equivalents required for the reduction are provided either by H_2 or by reduced coenzyme F_{420} ($F_{420}H_2$) in reactions catalyzed by F_{420}-non-reducing hydrogenase or $F_{420}H_2$-dehydrogenase, respectively. The reduction of CoM-S-S-CoB is catalyzed by heterodisulfide reductase (HDR), forming CoM-SH and CoB-SH (Figure 1) (Hedderich et al., 1998). The enzyme has been purified and characterized from *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain

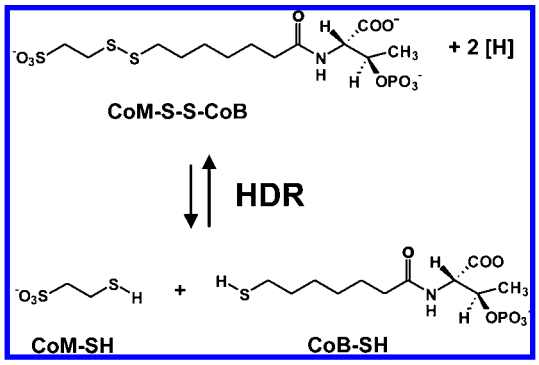

Figure 1 Reaction catalyzed by heterodisulfide reductase and structures of the coenzymes.

Marburg), *Methanosarcina barkeri* and *Methanosarcina thermophila* (Hedderich et al., 1990, 1994; Heiden et al., 1994; Künkel et al., 1997; Simianu et al., 1998). Reduction of CoM-S-S-CoB is reversible and HDR exhibits high specificity for its substrates CoM-S-S-CoB or CoM-SH plus CoB-SH.

HDR from *M. marburgensis* is an iron-sulfur flavoprotein that is composed of three different subunits, HdrA, B and C (Figure 2). From the primary sequence it was deduced that HdrA contains a typical FAD-binding motif and four binding motifs for [4Fe-4S] clusters. HdrB contains no characteristic cofactor-binding motif and HdrC contains two binding motifs for [4Fe-4S] clusters (Hedderich et al., 1994). HDR from the two closely related *Methanosarcina* species *M. barkeri* and *M. thermophila*

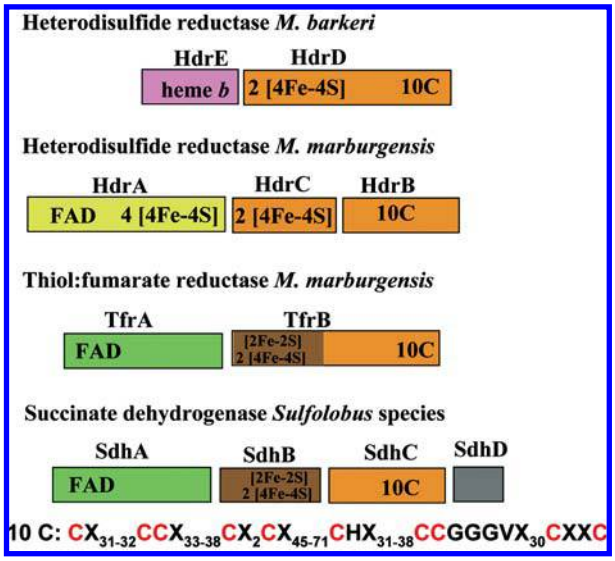

Figure 2 Schematic alignment of heterodisulfide reductase from *Methanosarcina barkeri*, heterodisulfide reductase from *Methanothermobacter marburgensis*, and thiol:fumarate reductase from *Methanothermobacter marburgensis.*
The subunits HdrD, HdrCB, and TfrB, which show a high degree of sequence similarity, are shown in light brown. In addition to the eight cysteine residues that coordinate the two [4Fe-4S] clusters, these subunits contain 10 conserved cysteine residues (10 C) in two $CX_{31-32}CCX_{33-38}CXXC$ sequence motifs. Subunits HdrE, HdrA, and TfrA have no sequence similarity and have different functions in the different enzymes. For comparison, succinate dehydrogenase from *Sulfolobus* species is shown. Subunit SdhC of this enzyme is homologous to HdrB.

is only composed of two subunits, a membrane-bound *b*-type cytochrome (HdrE) and a hydrophilic subunit (HdrD), the latter containing two classical binding motifs for [4Fe-4S] clusters (Figure 2) (Heiden et al., 1994; Künkel et al., 1997; Simianu et al., 1998).

The two types of HDR differ with respect to their physiological electron donor. *M. barkeri* HdrDE receives reducing equivalents from the reduced methanophenazine pool via its *b*-type cytochrome subunit. The enzyme is part of an energy-conserving membrane-bound electron transport chain with H_2 or reduced coenzyme F_{420} as electron donor and the heterodisulfide as terminal electron acceptor (Bäumer et al., 1998; Ide et al., 1999). *M. marburgensis* HdrABC forms a tight complex with the F_{420}-non-reducing hydrogenase (Mvh) (Setzke et al., 1994; Stojanowic et al., 2003). This six-subunit complex catalyzes the reduction of the heterodisulfide by H_2. For none of the subunits of this enzyme complex are transmembrane helices predicted with standard membrane-topology prediction programs, and after cell lysis this complex is almost completely localized in the soluble fraction. It is not yet known how this complex is attached to the membrane *in vivo* and how the exergonic reduction of the heterodisulfide is coupled to energy conservation in *M. marburgensis*.

For identification of the catalytic subunit of HDR, comparison between both enzymes was quite helpful. Subunit HdrD of the *M. barkeri* enzyme was shown to be a homologue of a hypothetical fusion protein of the *M. marburgensis* HdrCB subunits (Figure 2). A homologue of the *M. marburgensis* HdrA subunit is lacking in the enzyme from *Methanosarcina* species, while a homologue of the *Methanosarcina* HdrE subunit is lacking in the *M. marburgensis* enzyme. It has therefore been concluded that the conserved subunits HdrD and HdrCB must harbor the catalytic site for the reduction of the disulfide substrate, while the non-conserved subunits HdrE and HdrA interact with the physiological electron donor, which differs for the two types of HDR (Künkel et al., 1997). The proposed catalytic subunits *M. barkeri* HdrD and *M. marburgensis* HdrCB are also sequence-related to subunit TfrB of thiol:fumarate reductase from *Methanothermobacter* species, which catalyzes the reduction of fumarate to succinate with CoM-SH plus CoB-SH as electron donor, thus generating CoM-S-S-CoB (Figure 2) (Heim et al., 1998).

HdrB of *M. marburgensis* HDR contains two copies of a cysteine-rich sequence motif ($CX_{31-32}CCX_{33-38}CXXC$). This cysteine motif is also conserved in the C-terminal part of HdrD of *M. barkeri* HDR and in TfrB of thiol:fumarate reductase, as well as in a large number of other enzymes, e.g., succinate dehydrogenase from some archaeal species (Figure 2, Table 2). It has been proposed that part of these cysteine residues coordinate an additional iron-sulfur cluster (Künkel et al., 1997; Heim et al., 1998).

Characterization of HDR by EPR spectroscopy

The number, type and redox properties of Fe-S clusters in *M. marburgensis* HDR have been investigated by EPR and resonance Raman spectroscopies (Madadi-Kahkesh

et al., 2001). Oxidized HDR samples exhibited no signals attributable to paramagnetic Fe-S clusters and the resonance Raman spectrum was readily interpreted exclusively in terms of $S=0$ $[4Fe\text{-}4S]^{2+}$ clusters. In the absence of substrates, dye-mediated EPR redox titrations performed with HDR from *M. marburgensis* revealed a high-potential $[4Fe\text{-}4S]^{2+,+}$ center (E_m= -153 mV vs. NHE at pH 7.6) and multiple magnetically interacting low-potential $[4Fe\text{-}4S]^{2+,+}$ centers (E_m <-300 mV vs. NHE at pH 7.6). Similar results were obtained for redox titrations on HDR from *Methanosarcina thermophila* (Simianu et al., 1998), with the two $[4Fe\text{-}4S]^{2+,+}$ clusters having midpoint potentials of -100 and -400 mV.

EPR signals of potential relevance to the catalytic cycle were observed on reaction of oxidized HDR with either CoM-SH or CoB-SH, the co-substrates for the oxidative reaction (Madadi-Kahkesh et al., 2001). In the presence of CoM-SH, a novel $S=\frac{1}{2}$ resonance was observed at temperatures below 50 K, with principal *g* values of 2.013, 1.991 and 1.938 (Figure 3). This paramagnetic species has been designated CoM-HDR. The resonance was lost on reduction (E_m=-185 mV vs. NHE at pH 7.6) and on reaction with CoB-SH. Hence, it was attributed to the product of the oxidative half-reaction that occurs in the absence of CoB-SH, in which case it is likely to correspond to a trapped intermediate in the catalytic cycle. A species with similar *g* values (g=2.018, 1.996 and 1.954) and relaxation properties was observed when oxidized HDR was reacted with CoB-SH, designated CoB-HDR. However, redox titrations revealed a significantly higher midpoint potential (E_m=-30 mV vs. NHE at pH 7.6) for CoB-HDR than the CoM-SH-generated species and argue against a role as an intermediate in the HDR catalytic cycle. HDR from *M. barkeri* exhibited EPR spectroscopic properties very similar to those of the *M. marburgensis* enzyme after reaction of the oxidized enzyme with CoM-SH or CoB-SH (Madadi-Kahkesh et al., 2001). The CoM-SH-induced EPR signal in *M. marburgensis* HDR showed ^{57}Fe broadening, indicating that it corresponds to a paramagnetic iron-sulfur center (Madadi-Kahkesh et al., 2001).

To address the question as to whether CoM-SH is attached to this iron-sulfur center in CoM-HDR, CoM-^{33}SH was used as the substrate for the enzyme (Duin et al., 2003). With HDR from *M. marburgensis* and *M. barkeri*, anisotropic broadening of the EPR spectrum was obtained that results from anisotropic hyperfine coupling between the ^{33}S nucleus, which has a nuclear spin of $^3/_2$, and the paramagnetic center. From EPR simulations, hyperfine coupling constants of 7.0, 5.8, 1.8 G, and 7.0, 5.8 and 3.0 G were estimated for CoM-^{33}SH-treated *M. barkeri* and *M. marburgensis* HDR, respectively. The magnitude of this coupling suggests that the sulfur of CoM-SH is attached to an iron-site rather than a bridging sulfide.

Selenium K-edge X-ray absorption spectroscopy

To further investigate the site of CoM-SH binding to the cluster, studies with the substrate analog seleno-coenzyme M (CoM-Se$^-$) were performed. With HDR from *M. barkeri* and *M. marburgensis*, addition of CoM-Se$^-$ to the oxidized enzyme resulted in a new EPR spectrum with principal *g* values of 2.012, 1.993 and 1.974 (*M. marburgensis* HDR) and 2.012, 1.993 and 1.973 (*M. barkeri* HDR) (Figure 3; Duin et al., 2003). These signals could be observed under non-saturating conditions between 5 and 30 K. Kinetic studies confirmed that HDR catalyzes the oxidation of CoB-SH and CoM-Se$^-$ to CoM-Se-S-CoB, with duroquinone as electron acceptor. The finding that the substrate analog seleno-coenzyme M induces a new EPR signal is again indicative of direct binding of the compound to the active-site cluster of HDR.

To further elucidate the site of cluster attachment, selenium K-edge X-ray absorption spectroscopy (XAS) in combination with the selenium analog of coenzyme M (CoM-Se$^-$) was performed (Shokes et al., 2005). The same method had been used to determine the interaction between Se-adenosyl-L-selenomethionine and lysine 2,3-aminomutase (Cosper et al., 2000).

The best fit of the Se EXAFS data for HDR+CoM-SeH was a model that contains one Se-C bond (at 1.98 Å) and one Se-Fe interaction at ca. 2.4 Å. A search of the Cambridge Structural Database (Wavefunction Inc.) for Fe-Se-C fragments revealed a chemically reasonable average Se-Fe bond of 2.42±0.02 Å (Shokes et al., 2005, and references therein), consistent with the determination of 2.41 Å for HDR+CoM-SeH. These data provide a direct structural framework to interpret the EPR experiments on HDR incubated with the substrate CoM-^{33}SH (Duin et al., 2003). In particular, it provides direct structural evidence of an Fe-Se interaction in HDR reacted with CoM-SeH.

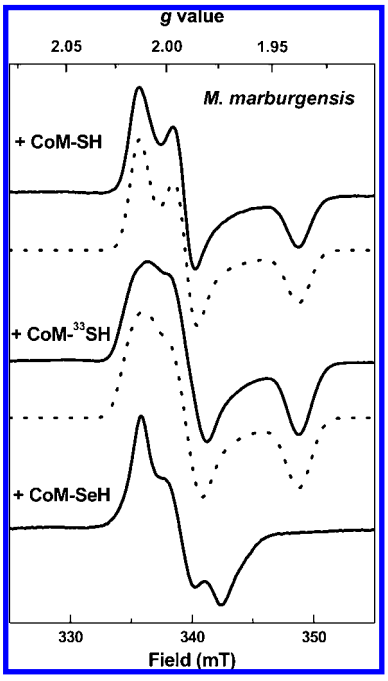

Figure 3 Comparison of EPR spectra of oxidized HDR from *M. marburgensis*.
The enzyme was treated with either CoM-SH containing the natural abundance mixture of sulfur, CoM-^{33}SH (more than 99% enriched in ^{33}S) or CoM-Se$^-$ (from Duin et al., 2003). The dotted lines are EPR simulations.

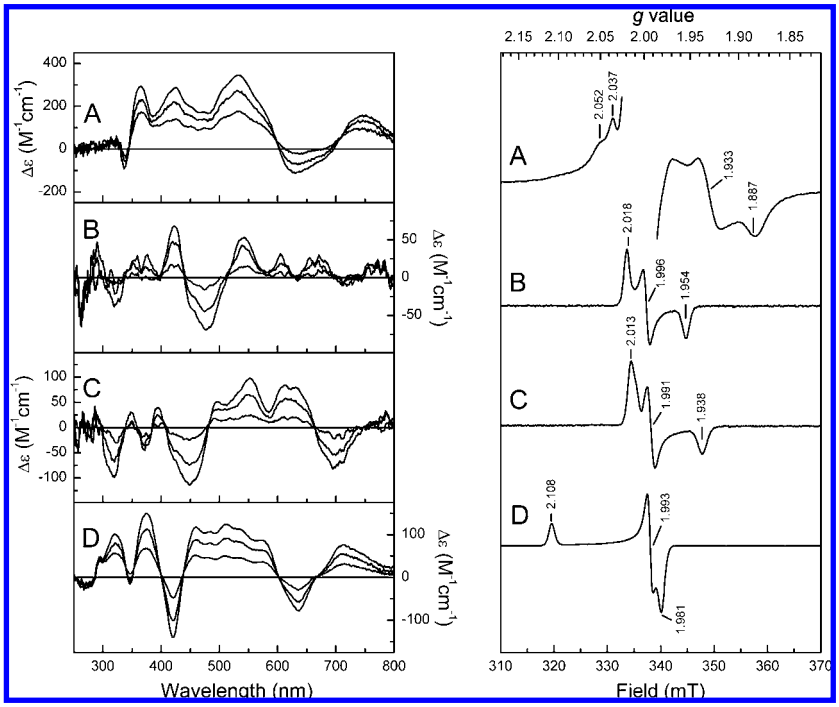

Figure 4 Comparison of the VTMCD and EPR spectra of different forms of HDR from *M. marburgensis*, and NEM-FTR from *Synechocystis* sp. PCC6803.
Left panel: VTMCD spectra recorded at 1.7, 4.2 and 10 K with an applied magnetic field of 6 T. All transitions increase in intensity with decreasing temperature. Right panel: EPR spectra recorded at 5 K (A) and 30 K (B–D) with 2 mW of microwave power and modulation amplitude of 0.6 mT at a microwave frequency of 9.43 GHz. (A) Dithionite-reduced HDR; (B) CoB-HDR, duroquinone-oxidized HDR incubated with CoB-SH; (C) CoM-HDR, duroquinone-oxidized HDR incubated with CoM-SH; (D) oxidized NEM-FTR (from Duin et al., 2002).

Characterization of the active-site Fe-S cluster in HDR by VTMCD spectroscopy

To elucidate the type of Fe-S cluster present in the active-site of HDR, the enzyme was also studied by variable-temperature magnetic circular dichroism (VTMCD; Duin et al., 2002). Only paramagnetic metal chromophores exhibit intense VTMCD bands and this allowed characterization of the ground- and excited-state properties of paramagnetic Fe-S centers in HDR samples, in which the absorption spectrum is dominated by contributions from diamagnetic Fe-S clusters and FAD (Johnson et al., 1999). CoM-HDR and CoB-HDR were generated by reacting the oxidized enzyme with either CoM-SH or CoB-SH. The absence of additional EPR signals at lower temperatures indicated that all the $[4Fe\text{-}4S]^{2+,+}$ clusters were oxidized and therefore diamagnetic (Madadi-Kahkesh et al., 2001). Hence, the VTMCD spectra of CoM-HDR (Figure 4C, left panel) and CoB-HDR (Figure 4B, left panel) are expected to facilitate selective characterization of the electronic transitions associated with the Fe-S centers responsible for these novel EPR signals. This was confirmed by variable-temperature, variable-field MCD saturation magnetization studies, which indicated that each of the temperature-dependent MCD bands of CoM-HDR and CoB-HDR originated exclusively from the $S=\frac{1}{2}$ ground state responsible for the EPR signals. The VTMCD spectra of both CoB-HDR and CoM-HDR were different from each other and both were distinct from those associated with the most common types of oxidized paramagnetic Fe-S clusters, i.e., cubane and linear $[3Fe\text{-}4S]^{+}$ clusters and HiPIP-type $[4Fe\text{-}4S]^{3+}$ clusters (Johnson et al., 1999). However, the VTMCD spectra of CoM-HDR and CoB-HDR both showed correspondence to that observed for the novel type of $[4Fe\text{-}4S]^{3+}$ cluster found in *N*-ethylmaleimide-modified ferredoxin:thioredoxin reductase (NEM-FTR; see below) (Staples et al., 1996, 1998; Figure 4D, left panel). The similarity was particularly striking on comparison of the VTMCD spectra of CoM-HDR and NEM-FTR. Both have the same pattern of positive and negative bands, with equivalent bands shifted to lower energy by approximately 2000 cm^{-1} in CoM-HDR. The correspondence is less immediately apparent between the VTMCD spectra of CoB-HDR and NEM-FTR due to changes in the relative intensities of the MCD bands and a further red shift of each of the bands. Nevertheless, the pattern of VTMCD bands observed in NEM-FTR is clearly preserved in both CoM-HDR and CoB-HDR, indicating that each contains a similar type of novel $[4Fe\text{-}4S]^{3+}$ cluster.

^{57}Fe-ENDOR evidence for an unusual [4Fe-4S] cluster in the active-site of HDR

^{57}Fe pulsed ENDOR spectroscopy was used to identify the iron sites of the active cluster (Bennati et al., 2004) and to gain more insight into the structure of the CoM-HDR paramagnetic intermediate. The experiments were performed at two very different frequencies, 9 and 94 GHz, and consistently showed two pairs of doublets

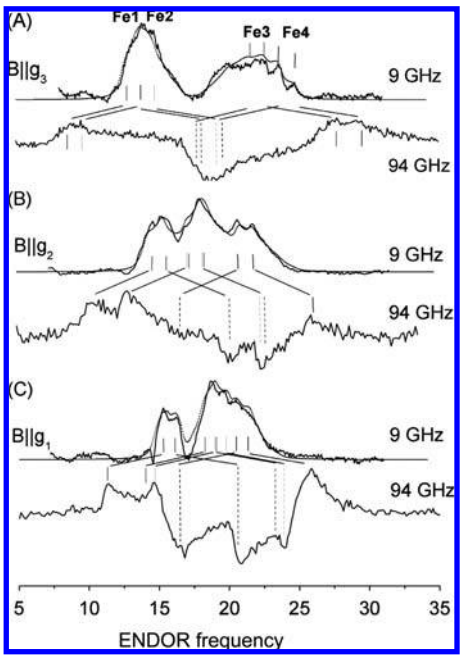

Figure 5 Davies ENDOR spectra of ^{57}Fe-enriched CoM-HDR recorded at 9 and 94 GHz at different positions of the EPR line according to $B||g_3$ (A), $B||g_2$ (B) and $B||g_1$ (C). Simulations are displayed as dotted lines. The 9-GHz spectra were recorded by strongly attenuating the overlapping ^{1}H resonances with hard microwave preparation pulses. The remaining features of the ^{1}H resonances were subtracted after a reference measurement (from Bennati et al., 2004).

arising from four distinct iron sites. The multi-frequency approach allowed quantitative analysis with spectral simulation and assignment of all resonances observed. The spectra recorded at the three canonical positions of the EPR line (i.e., B parallel to g_3, g_2 and g_1) are displayed in Figure 5, together with the simulations. Furthermore, the ENDOR spectra at 94 GHz displayed absorptive and emissive lines due to thermal electron-spin polarization. The pattern observed allowed the sign of the hyperfine coupling at each iron site to be determined.

The hyperfine tensors obtained from the simulation and from the sign of the hyperfine couplings gave rise to the isotropic hyperfine constants reported in Table 1. For comparison, we also report the hyperfine tensors of a typical four-fold cysteine-coordinated $[4Fe\text{-}4S]^{3+}$ cluster in a model compound (Rius and Lamotte, 1989), which is very similar to those found in HiPIP proteins (Kappl et al., 1999). We also used the hyperfine tensors obtained for NEM-FTR for comparison (Jameson et al., 2003). The ^{57}Fe couplings of the $[4Fe\text{-}4S]^{3+}$ model compound and of the HiPIP protein are systematically lower than in CoM-HDR. Thus, the results suggest that the couplings in CoM-HDR reflect the interaction of the cluster with the substrate, which is absent in HiPIP proteins or model compounds. Enhanced ^{57}Fe couplings were also observed in NEM-FTR and could be indicative of the similarity between the two cluster species, as proposed from the previous EPR (Madadi-Kahkesh et al., 2001; Duin et al., 2003) and VTMCD (Duin et al., 2002) data. Nevertheless, in NEM-FTR, pronounced anisotropy (ca. 66%) of the ^{57}Fe coupling was reported at a unique site (Jameson et al., 2003), which was not observed in CoM-HDR (Bennati et al., 2004).

Table 1 Isotropic ^{57}Fe hyperfine couplings of CoM-HDR (Bennati et al., 2004) compared to a $[4Fe\text{-}4S]^{3+}$ cluster in a model system from a single crystal ENDOR study (Rius and Lamotte, 1989), in a HiPIP protein (Kappl, 1999) and to the cluster intermediate in NEM-FTR (Jameson et al., 2003).

Fe site	a_{iso} (MHz)			
	Model system	HiPIP protein	NEM-FTR	CoM-HDR
1	17.4	21.6	22	29
2	19.8	21.6	27	33.3
3	-32.7	-33	-37	-39.2
4	-33.5	-33	-37	-43.4

The data for NEM-FTR were extracted from the Mössbauer data.

Comparative studies with ferredoxin:thioredoxin reductase (FTR)

FTR catalyzes the reduction of a disulfide on the protein substrate, thioredoxin, in two one-electron steps, using reduced [2Fe-2S] ferredoxin as the electron donor. The enzyme is found in chloroplasts and cyanobacteria and plays a central role in light regulation of the activity of enzymes involved in oxygenic photosynthesis (Walters and Johnson, 2004).

FTR is a $\alpha\beta$-heterodimer composed of a highly conserved 13-kDa catalytic β-subunit and a variable α-subunit of similar or smaller mass. The catalytic subunit contains a redox-active disulfide and a [4Fe-4S] cluster as the sole prosthetic group (Droux et al., 1987). Extensive spectroscopic studies using EPR, resonance Raman, VTMCD, ENDOR and Mössbauer spectroscopies have been performed with *N*-ethylmaleimide-modified FTR (NEM-FTR) from spinach, in which one of the cysteines that form the redox-active disulfide is selectively alkylated (Staples et al., 1996, 1998). NEM-FTR was found to be paramagnetic in the oxidized state and exhibited a near-axial EPR signal (g=2.112, 1.997 and 1.984 for spinach NEM-FTR). The resonance was lost upon dithionite reduction in a reversible one-electron process, with a midpoint potential of -210 mV. Under turnover conditions, non-alkylated FTR showed the same EPR signal as observed for the cluster in NEM-FTR, indicating that the latter species is a stable analog of a one-electron-reduced enzymatic intermediate. Spectroscopic characterization of the paramagnetic species observed in NEM-FTR strongly indicates the presence of a $[4Fe\text{-}4S]^{3+}$ cluster with anomalous EPR and MCD properties and with a 500–600-mV decrease in redox potential compared to the $[4Fe\text{-}4S]^{3+}$ cluster in oxidized HiPIPs. Furthermore, Mössbauer experiments (Jameson et al., 2003) revealed ^{57}Fe hyperfine couplings, reported here in Table 1, which indicated unusually large anisotropy at only one Fe site. These anomalous properties have been interpreted in terms of a $[4Fe\text{-}4S]^{3+}$ cluster with a unique five-coordinate Fe site with two cysteinate ligands. In the catalytic cycle, one-electron reduction of the redox-active disulfide generates a thiol and a thiyl radical, with the latter stabilized by reduction and coordination to the

[4Fe-4S] cluster and generating the unique $[4Fe\text{-}4S]^{3+}$ center.

The crystal structure is known for FTR from *Synechocystis* sp. PCC6803. The structure shows a cysteine disulfide asymmetrically positioned relative to the cluster, with one of the S atoms at a distance of 3.1 Å from both a cluster Fe and the S atom of a cluster-ligating cysteinate, and 3.5 Å from a cluster μ_3-S atom. The crystal structure suggests that a cluster Fe is the most likely point of attachment to yield an intermediate involving a [4Fe-4S] cluster with a five-coordinate Fe site (Dai et al., 2000).

For HDR, several lines of evidence argue in favor of a mechanism involving direct interaction of the heterodisulfide substrate with the active-site [4Fe-4S] cluster rather than the FTR-type mechanism, in which cleavage of the substrate disulfide by the [4Fe-4S] cluster is mediated by an active-site disulfide in close proximity to the cluster. First, the experiments with CoM-^{33}SH provide strong evidence for direct binding of the substrate to the cluster. Second, the $[4Fe\text{-}4S]^{3+}$ species in HDR are readily formed under oxidizing conditions on addition of exogenous thiols such as CoM-SH, CoB-SH, DTT or β-mercaptoethanol (Madadi-Kahkesh et al., 2001). This does not occur in FTR, since the active-site disulfide that is present in oxidized samples can only be cleaved under reducing conditions using the physiological electron donor, reduced ferredoxin, or mediator dyes such as reduced viologens (Schürmann et al., 1995). The $[4Fe\text{-}4S]^{3+}$ species in FTR is only observed as a stable species on oxidation when one of the active-site cysteine residues has been alkylated, and therefore is not available to reform the active-site disulfide on oxidation, leaving the free cysteine available to interact with the cluster. Third, *M. marburgensis* Hdr is not inhibited by cysteine alkylating reagents at concentrations up to 2 mM (Madadi-Kahkesh et al., 2001), whereas cysteine alkylating reagents are potent inhibitors of FTR as a result of alkylation of the interchange thiol of the active-site disulfide (Staples et al., 1996).

Subunit HdrB of *M. marburgensis* HDR contains the active-site iron-sulfur cluster

To elucidate if the unique cysteine motif present in HdrB and in the C-terminal part of HdrD (Figure 2) binds the active-site iron-sulfur cluster, HdrB was produced in a heterologous host. HdrB was chosen since it does not contain a classic [4Fe-4S] cluster-binding motif (CxxCxxCxxxCP), which could interfere with the spectroscopic characterization. Initial expression experiments of *hdrB* in *B. subtilis* and *E. coli* resulted in a brown protein with a content of 2 mol non-heme iron and 2 mol of acid-labile sulfur per mol protein (Madadi-Kahkesh et al., 2001). UV/visible and resonance Raman (RR) spectroscopies showed that HdrB thus produced contains a [2Fe-2S] cluster. This cluster was, however, labile and was destroyed upon reduction by sodium dithionite, as indicated by the loss of absorbance in the visible region. The RR spectrum of as-isolated HdrB showed an absorption band at 290 cm^{-1} characteristic for [2Fe-2S] clusters. In native HDR, this absorption band at 290 cm^{-1} was not observed. It was therefore concluded that the [2Fe-2S] cluster found in HdrB is an artifact of the heterologous expression system. When, however, the heterologously produced protein was incubated *in vitro* under anaerobic conditions in the presence of Fe^{2+}, Na_2S and cysteine, the UV/visible absorption spectrum with maxima at 320, 420 and 460 nm changed to a spectrum with a broad absorbance between 350 and 500 nm. Furthermore, reduction of the cluster by sodium dithionite was now reversible and did not result in cluster breakdown. This protein, when oxidized, exhibited an EPR signal with g=2.016, 1.995 and 1.952 reminiscent of the CoB-HDR signal (Hamann and Hedderich, unpublished results). In contrast to the native enzyme (HdrABC), which was EPR-silent when oxidized in the absence of its thiol substrates, HdrB showed the characteristic EPR signal upon oxidation of the protein even in the absence of either CoB-SH or CoM-SH. This result is puzzling, since it indicates that HdrB is able to generate a cluster with similar electronic and structural properties in the absence of substrate. However, native HDR also exhibited a CoM-HDR-like EPR signal when oxidized in the presence of non-substrate thiols such as mercaptoethanol or cysteine. These paramagnetic species, as CoB-HDR, had redox potentials at least 170 mV more positive than CoM-HDR. They may be formed in an artificial side reaction occurring at high redox potential. Since no extra thiol was added during sample preparation of HdrB, it has to be considered that the protein uses an intrinsic cysteine residue as an extra thiol ligand to form the paramagnetic center upon oxidation. HdrB did not catalyze the reduction of CoM-S-S-CoB with reduced benzylviologen as electron donor, nor the oxidation of CoM-SH plus CoB-SH with methylene blue as electron acceptor. A possible reason could be that HdrC is required for electron transfer to these redox dyes. Although the paramagnetic species present in HdrB needs to be further characterized (e.g., by ENDOR spectroscopy), the data clearly show that subunit HdrB binds the active-site iron-sulfur cluster, most probably involving the unique Cys-motif (Hamann and Hedderich, unpublished results).

Proposed catalytic mechanism for HDR

Results obtained from the spectroscopic characterization of CoM-HDR led to the proposal of a mechanistic scheme for the reversible heterodisulfide/dithiol cleavage reaction that is catalyzed by HDR in two one-electron steps (Figure 6; Duin et al., 2002). The reaction cycle is described here for the oxidative reaction. This mechanism involves an EPR-silent $[4Fe\text{-}4S]^{2+}$ in the resting enzyme (**1**). Binding of CoM-SH (**2**) to the active site in the presence of an electron acceptor generates the paramagnetic reaction intermediate CoM-HDR (**3**). Spectroscopic data clearly indicate that CoM-HDR is a $[4Fe\text{-}4S]^{3+}$ cluster, with CoM-S^- attached to the cluster. EPR studies with CoM-^{33}SH and Se-X-ray absorption spectroscopic studies with the substrate analog CoM-

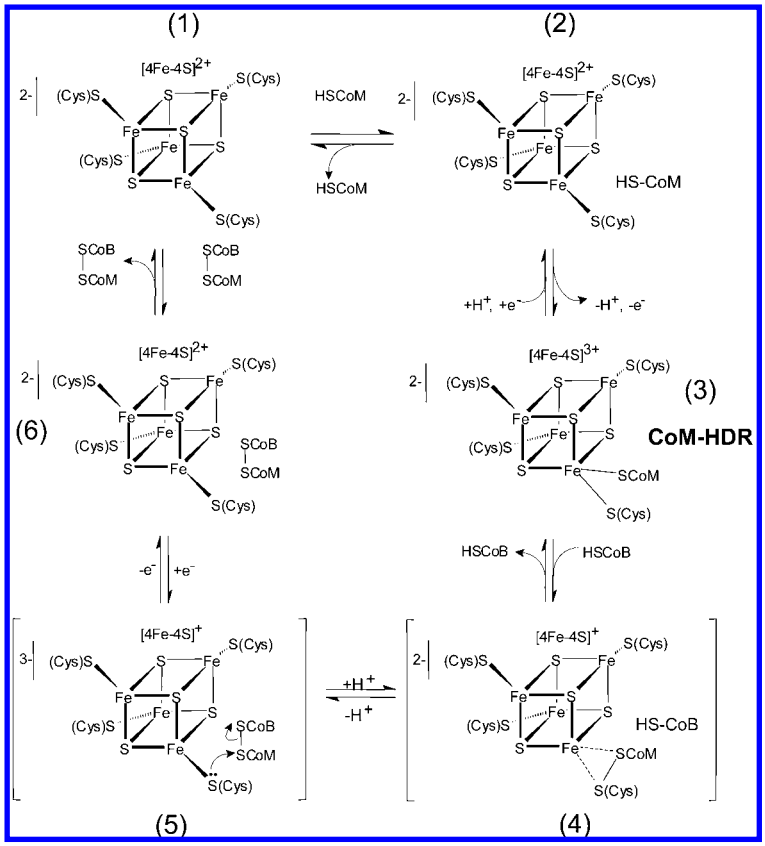

Figure 6 Proposed active-site mechanism for the reversible heterodisulfide/dithiol cleavage reaction catalyzed by HDR.
The oxidation state of the [4Fe-4S] cores is indicated above each of the cubane clusters and the charge of the cluster including the thiolate ligands is indicated to the left of the lines next to the clusters. The different reaction intermediates are numbered. Transient intermediates are shown in brackets and the electron arrows on one of the transient intermediates correspond to the reductase reaction (from Duin et al., 2002). The stable paramagnetic reaction intermediate is labeled as CoM-HDR.

SeH showed that CoM-S^- binds to an iron site of the cluster rather than to a cluster μ_3-S. In formal terms, the reaction involves the oxidation of CoM-S^- to a thiyl radical, which is stabilized by coordination to the $[4Fe\text{-}4S]^{2+}$ cluster, resulting in the formation of a $[4Fe\text{-}4S]^{3+}$ cluster with a CoM-S^- ligand. At this stage, it is unclear whether the CoM-S^- ligand binds at a unique iron site, replacing one cysteine ligand or generating a five-fold coordinating iron [as shown in Figure 6 (**3**)]. ^{57}Fe couplings from ENDOR data compared to NEM-FTR and the observation of a cluster species similar to CoM-HDR in isolated HdrB, in the absence of substrate, would be more consistent with the former hypothesis. Nevertheless, more experiments are required to clarify this point. When CoB-SH is added to the CoM-HDR reaction intermediate (**4** and **5**) in the presence of an electron acceptor, this results in the formation of the diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster and CoM-S-S-CoB (**6**). In this series of events, CoB-SH attacks the CoM-thiol group in CoM-HDR in a nucleophilic substitution reaction, forming the disulfide and the cluster in the 1+ reduced state (**5**). It is not known if the active-site $[4Fe\text{-}4S]^{2+}$ cluster is reducible to a stable $[4Fe\text{-}4S]^{+}$ state or if it is immediately reoxidized by the [4Fe-4S] centers in HdrC. The evidence that CoM-S^- rather than CoB-S^- binds to the cluster is based on the observation that the CoM-HDR EPR signal is lost on addition of CoB-SH and that the redox potential for CoM-HDR ($E^{\circ\prime}$=-185±10 mV vs. SHE) is close to the heterodisulfide/dithiol couple ($E^{\circ\prime}$=-143±10 mV vs. SHE; Tietze et al., 2003).

The catalytic subunit of HDR contains a unique Cys-motif conserved in a large number of proteins

HdrB of *M. marburgensis* HDR contains two copies of the unique $CX_{31\text{-}32}CCX_{33\text{-}38}CXXC$ sequence motif, which are also conserved in HdrD of *M. barkeri* HDR and in subunit TfrB of thiol:fumarate reductase (Figure 2). A database search revealed a large number of proteins from non-methanogenic microorganisms with high sequence similarity to HdrB, HdrD and TfrB (Table 2). In these proteins at least one of the two $CX_{31\text{-}32}CCX_{33\text{-}38}$ CXXC sequence motifs is conserved. For many of these HDR-related enzymes the catalytic function is not known and they were only identified through genome sequencing projects. Some of these proteins are more closely related to HdrB and lack additional cofactor binding motifs; others are more closely related to HdrD and contain, as HdrD, two four-Cys motifs characteristic for the binding of two [4Fe-4S] clusters in the amino-terminal part of the protein. More detailed biochemical studies have been performed with HDR-related enzymes from

Table 2 Gene products with significant sequence similarity to the postulated catalytic subunit HdrD of the *M. barkeri* heterodisulfide reductase, the postulated catalytic subunits HdrCB of the *M. thermoautotrophicum* heterodisulfide reductase and the subunit TfrB of thiol:fumarate reductase.

Gene	Organism	Comment/putative transcriptional unit	Sequence identity to	Reference
AF 0506	*Archaeoglobus fulgidus*	–	HdrD (34% from 385 aa)	Klenk et al., 1997
AF 0547	*Archaeoglobus fulgidus*	Putative operon with a gene encoding a *b*-type cytochrome	HdrD (23% from 431 aa)	Klenk et al., 1997
AF 0502	*Archaeoglobus fulgidus*	Hme complex, operon with genes encoding Fe/S proteins and *b*-type cytochromes	HdrD (32% from 149 aa)	Mander et al., 2002
AF 0543	*Archaeoglobus fulgidus*	Most similar to *hmc6* from *D. vulgaris*	HdrD (20% from 354 aa)	Klenk et al., 1997
AF 0544	*Archaeoglobus fulgidus*	Most similar to *hmc6* from *D. vulgaris*	HdrD (20% from 338 aa)	Klenk et al., 1997
AF 1375	*Archaeoglobus fulgidus*	Mvh:Hdl complex	HdrB (30% from 300 aa)	Mander et al., 2004
ywjF	*Bacillus subtilis*	Putative operon with *acdA* encoding acyl-CoA dehydrogenase	HdrD (28% from 390 aa)	Kunst et al., 1997
hdrD	*Aquifex aeolicus*	Hydrogenase operon	HdrD (25% from 409 aa)	Deckert et al., 1998
hdrB	*Aquifex aeolicus*	*hdrABC* operon	HdrB (30% from 300 aa)	Deckert et al., 1998
isp2	*Thiocapsa roseopersicina*	Hydrogenase operon	HdrD (23% from 398 aa)	Rakhely et al., 1998
sdhC	*Sulfolobus acidocaldarius*	Succinate dehydrogenase operon	HdrB (31% from 277 aa)	Janssen et al., 1997
hdrB	*Synechocystis* sp.	–	HdrB (33% from 300 aa)	Nakamura et al., 1998
hmc6	*Desulfovibrio vulgaris*	*hmc* operon; encodes a multisubunit membrane complex	HdrD (33% from 103 aa)	Rossi et al., 1993
dsrK	*Allochromatium vinosum*	dsr locus encoding sulfite reductase	HdrD (23% from 437 aa)	Pott and Dahl, 1998
glpC	*Escherichia coli*	Subunit of anaerobic glycerol-3-phosphate dehydrogenase	HdrD (27% from 251 aa)	Cole et al., 1988
glcF	*Escherichia coli*	Subunit of glycolate oxidase		Pellicer et al., 1996

Note that most of the genes are derived from genome sequencing projects and that the function of these genes and their gene products are unknown. The table only gives a selection of proteins or gene products. The conserved domain present in these proteins has been designated as domain of unknown function (DUF) 224 in the Interpro database.

Archaeoglobus species, which are sulfate-reducing archaea. Membrane-bound enzymes have been isolated from *A. fulgidus* and *A. profundus*, designated Hme (HDR-like menaquinol-oxidizing enzyme), that contain a HdrD-related subunit (Mander et al., 2002, 2004). Hme is membrane-bound and functions as menaquinol:acceptor oxidoreductase. Hme, when oxidized, exhibits an EPR signal with similar *g* values (e.g., $g=2.031$, 1.995, and 1.951 for *A. fulgidus* Hme) and relaxation properties to CoM-HDR or CoB-HDR. This paramagnetic species could be reduced in a one-electron reduction step to a diamagnetic form with a midpoint potential of +100 mV. A second enzyme complex was isolated from *A. profundus*, designated Mvh:Hdl complex, containing a HdrB-related subunit (Mander et al., 2004). In the oxidized state the enzyme complex exhibited a rhombic EPR signal with $g_{xyz}=2.014$, 1.939 and 1.895. In these HDR-like enzymes the characteristic CoM-HDR-like EPR spectrum is generated upon oxidation of the enzymes, even in the absence of substrate. This is a major difference to HDR. The physiological substrate of these enzymes is not known. It has been proposed that they reduce an as yet unknown disulfide substrate, which could then donate reducing equivalents to the enzymes of sulfate reduction. CoM-SH and CoB-SH are not present in *Archeoglobus* species. Hme-related enzymes are also present in sulfate-reducing bacteria, e.g., *Desulfovibrio* species (Rossi et al., 1993).

More detailed biochemical studies have also been performed with some unusual archaeal succinate dehydrogenases, e.g., the enzymes from *Sulfolobus acidocaldarius* and *Acidianus ambivalens* (Janssen et al., 1997; Lemos et al., 2001). These enzymes contain a subunit (SdhC) that is sequence-related to HdrB. They lack, however, the characteristic heme *b*-containing membrane-anchor protein found in other succinate dehydrogenases and fumarate reductases. It has therefore been proposed that in these enzymes SdhC functions as a membrane anchor that reduces the quinone of these organisms (Lemos et al., 2001). Production of SdhC from *Sulfolobus tokodaii* strain 7 in *E. coli* resulted in a protein containing a [2Fe-2S] cluster, which was, however, destroyed upon reduction (Iwasaki et al., 2002). The same properties were found for HdrB when produced in *E. coli* as outlined above. In the case of HdrB, a protein that most likely contains a [4Fe-4S] cluster was only obtained after *in vitro* reconstitution. This might also be true for SdhC.

A common feature of these HdrB- and HdrD-related proteins could be their ability to ligate a [4Fe-4S] cluster using the unique Cys-motif. In some enzymes this cluster could function as a one-electron/two-electron switch similar to flavins, and could thus catalyze the reduction of a disulfide in two one-electron reduction steps. In other enzymes, such as the archaeal succinate dehydrogenase, it is very unlikely that the enzyme reduces a disulfide. Here, reduction of a quinone is the most likely function. Given the high similarity between these succinate dehydrogenases and thiol:fumarate reductase from methanogens, it is also conceivable that archaeal succinate dehydrogenases are evolutionarily derived from thiol:fumarate reductase. Hence, the carboxyterminal domain of subunit TfrB of thiol:fumarate reductase (Figure 2) may have been converted into a quinone-reducing subunit when aerobic respiration became available.

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