Characterization of the Intramolecular Electron Transfer Pathway from 2-Hydroxyphenazine to the Heterodisulfide Reductase from *Methanosarcina thermophila*

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Heterodisulfide reductase (HDR) is a component of the energy-conserving electron transfer system in methanogens. HDR catalyzes the two-electron reduction of coenzyme B-S-S-coenzyme M (CoB-S-S-CoM), the heterodisulfide product of the methyl-CoM reductase reaction, to free thiols, HS-CoB and HS-CoM. HDR from *Methanosarcina thermophila* contains two b-hemes and two [Fe₄S₄] clusters. The physiological electron donor for HDR appears to be methanophenazine (MPhen), a membrane-bound cofactor, which can be replaced by a water-soluble analog, 2-hydroxyphenazine (HPhen). This report describes the electron transfer pathway from reduced HPhen (HPhenH₂) to CoB-S-S-CoM. Steady-state kinetic studies indicate a ping-pong mechanism for heterodisulfide reduction by HPhenH₂ with the following Michaelis-Menten values:

\[ k_{cat} = 74 \text{ s}^{-1} \text{ at } 25 \degree C, K_m (\text{HPhenH}_2) = 92 \mu M, K_m (\text{CoB-S-S-CoM}) = 144 \mu M. \]

Rapid freeze-quench EPR and stopped-flow kinetic studies and inhibition experiments using CO and diphenylene iodonium indicate that only the low spin heme and the high potential FeS cluster are involved in CoB-S-S-CoM reduction by HPhenH₂. Fe-S cluster disruption by mersalyl acid inhibits heme reduction by HPhenH₂, suggesting that a 4Fe cluster is the initial electron acceptor from HPhenH₂. We propose the following electron transfer pathway: HPhenH₂ to the high potential 4Fe cluster, to the low potential heme, and finally, to CoB-S-S-CoM.

Heterodisulfide reductase (HDR) plays important roles in methane production by methanogenic archaea. In the last step of methanogenesis, the methyl group of methylated coenzyme M (2-mercaptoethane sulfonic acid, HS-CoM) is reduced to methane by methyl-CoM reductase reductase. Coenzyme B (7-mercaptoheptanoyl-threonine phosphate, HS-CoB) is the initial electron acceptor from HPhenH₂. The physiological electron donor for HDR appears to be methanophenazine (MPhen), a membrane-bound cofactor that can be replaced by a water-soluble analog, 2-hydroxyphenazine (HPhen). This report describes the electron transfer pathway from reduced HPhen (HPhenH₂) to CoB-S-S-CoM. Steady-state kinetic studies indicate a ping-pong mechanism for heterodisulfide reduction by HPhenH₂ with the following Michaelis-Menten values:

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\[ \text{CH}_3\text{CoM} + \text{HS-CoB} \rightarrow \text{CH}_4 + \text{CoB-S-S-CoM} \] (Eq. 1)
\[ \text{CoB-S-S-CoM} + 2e^- + 2H^+ \rightarrow \text{HS-CoB} + \text{HS-CoM} \] (Eq. 2)

The ultimate electron donor to HDR can be the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex when bacteria grow on acetate. In this case, the electron transfer pathway involves ferredoxin and possibly an iron-sulfur flavoprotein (5, 6). When bacteria grow on methanol, the ultimate donor is reduced coenzyme F₄₂₀H₂ generated by F₄₂₀ dehydrogenase. The F₄₂₀ dehydrogenases from *Methanosarcina mazei* (7), *Methanolobus tindarius* (8), and *Archaeoglobus fulgidus* (9) have been isolated and shown to contain Fe-S clusters and FAD. The direct electron donor for HDR appears to be a membrane-bound cofactor, methanophenazine (MPhen) (10). This cofactor has been isolated from membranes of *M. mazei* strain G61 (11) and *Methanosarcina thermophila*.² It has a 25-carbon isoprenoid chain attached to position 2 of phenazine via an ether bond, which makes it insoluble in aqueous solution (11). The 2-hydroxyphenazine (HPhen) derivative is a suitable water-soluble substitute for MPhen that can accept electrons from F₄₂₀H₂ and can donate electrons to the purified HDR from *M. thermophila*. (10). Electron transfer from F₄₂₀H₂ to HPhen results in the translocation of two protons per two electrons transferred (12). Another two protons (per two electrons) are translocated during reduction of CoB-S-S-CoM by HPhen (13).

HDR from *M. thermophila* consists of two subunits. A 53-kDa subunit contains two distinct [Fe₄S₄] clusters with midpoint potentials of −100 and −400 mV (6). A 27-kDa membrane-associated subunit contains two b-heme types, one that is low spin and is hexacoordinate and another that is high spin and is five-coordinate. The midpoint potentials of the low and high spin hemes are −180 and −23 mV, respectively (6).

We have used steady-state and pre-steady-state kinetics to answer some key questions about the HDR mechanism. Which of the metal centers in HDR is the initial electron acceptor from reduced HPhen (HPhenH₂)? The midpoint potentials of some of the metal centers are outside the range of the HPhen/HPhenH₂ and CoB-S-S-CoM/CoB-S-S-CoM couples; therefore, are all of the metal clusters involved in the electron transfer reaction? What is the intramolecular electron transfer pathway? Based on our results, we propose that the physiological electron transfer pathway from methanophenazine to the heterodisulfide is:

\[ \text{MPhenH}_2 \rightarrow [\text{Fe}_4\text{S}_4]_{\text{high}} \rightarrow \text{heme}_{\text{low}} \rightarrow \text{CoB-S-S-CoM}. \]

² U. Deppenmeier, E. Murakami, and S. W. Ragsdale, manuscript in preparation.

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1 The abbreviations used are: HDR, heterodisulfide reductase; HS-CoM, coenzyme M or 2-mercaptoethane sulfonic acid; HS-CoB, coenzyme B or 7-mercaptoheptanoyl-threonine phosphate; MPhen, methanophenazine; HPhen, 2-hydroxyphenazine; DPI, diphenylene iodonium.

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**Electron Transfer in Heterodisulfide Reductase**

**Fig. 1. Steady-state kinetics of the reduction of CoB-S-S-CoM by HPhenH$_2$.** The rate of HPhenH$_2$ oxidation was followed at 365 nm ($\Delta A_{\text{obs}} = 3.58 \text{ mm}^{-1} \text{ cm}^{-1}$). The concentration of CoB-S-S-CoM was varied at fixed HPhenH$_2$ concentrations of 200 (closed circles), 100 (open circles), 50 (closed triangles), and 20 $\mu$M (open triangles). The data were globally fit to the equation for a two-substrate ping-pong mechanism (see "Experimental Procedures") to yield the following Michaelis parameters: $V_{\text{max}} = 54 \pm 7$ units/mg, $K_m$ for phenazine $= 93 \pm 28$ $\mu$M, and $K_m$ for CoB-S-S-CoM $= 296 \pm 65$ $\mu$M. Inset: plot of $V_{\text{max}}/K_m$ for CoB-S-S-CoM at different concentrations of HPhenH$_2$.

**Fig. 2. Stopped-flow kinetics of the reduction of HDR by HPhenH$_2$.** Thionin-oxidized HDR (6 $\mu$M before mixing) was rapidly mixed with HPhenH$_2$ (10, 20, 50, 100, 150, and 200 $\mu$M before mixing). Reduction of the hemes was followed at 423 nm. Two phases with equal amplitude were observed, a fast phase with $k_{\text{obs}} = 87 \text{ s}^{-1}$ (○) and a slow phase with a rate constant of 9.4 $\text{s}^{-1}$ (□).

**EXPERIMENTAL PROCEDURES**

**Materials—**HPhen was synthesized as described (11). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo).

**Cell Growth and Enzyme Preparation—** *M. thermophila* TM1 was cultured on acetate at 50 °C and pH 6.8 in a 5-liter New Brunswick fermentor equipped with a pH auxostat (14) as described (15). HDR was purified as described previously (6), except for enzyme concentration steps. As mentioned by Thauer et al. (16), higher activity was recovered when membranes with molecular/pore molecular mass cut-off of 50 kDa (Spectrum) were used.

**Enzyme Assays—** HDR activity was measured by monitoring the oxidation of reduced methyl viologen at 604 nm ($\epsilon = 13.9 \text{ mm}^{-1} \text{ cm}^{-1}$) and 55 °C (6, 17, 18). One unit of HDR activity corresponds to 1 $\mu$mol of CoB-S-S-CoM reduced per minute. HDR activity was also assayed using 2-hydroxyphenazine as the electron donor. A solution containing HPhen (final concentration of 200 $\mu$M) in Buffer A (50 mM Tris, pH 7.6, and 10% glycerol) was reduced by bubbling with 100% hydrogen gas for 20 min, adding partially purified hydrogenase from *M. thermophila*, and incubating at 55 °C for 2 h. For steady-state kinetic experiments, varying amounts of the HPhenH$_2$ stock solution were added to Buffer A. Then, HDR was added and the reaction was started by adding CoB-S-S-CoM. The oxidation of HPhenH$_2$ was monitored either at 25 °C or at 55 °C by following an increase in absorbance at 365 nm. The difference (oxidized minus reduced, $\Delta\epsilon$) extinction coefficient at 365 nm was measured to be 3.58 $\text{mm}^{-1} \text{ cm}^{-1}$. One unit is defined as 1 $\mu$mol of CoB-S-S-CoM reduced min$^{-1}$ mg$^{-1}$. The data were fit to eq. 3 for a ping-pong reaction, where $v_0$ is initial velocity and $A$ and $B$ are HPhenH$_2$ and CoB-S-S-CoM, respectively, as follows.

$$v_0 = V_{\text{max}}/B_0/[K_m^A + [A]/[B]]$$

(Eq. 3)

Protein concentrations were determined by the Bio-Rad protein assay (19) using bovine serum albumin as a standard. UV-visible spectra were collected on a Cary-14 spectrophotometer modified by On-Line Instrument Systems, Inc. (Bogart, GA). EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a temperature controller (Oxford ITC4) and automatic frequency counter (Model 5340A, Hewlett-Packard Co.).

**Stopped-flow Experiments—** HDR was oxidized by adding thionin ($E'_o = +60 \text{ mV}$) until the blue color disappeared. Excess thionin was removed by passing the solution through a Sephadex G-25 column (Amersham Pharmacia Biotech). Oxidized HDR (6 $\mu$M before mixing)
and varied concentrations of HPhenH2 (10, 20, 50, 100, 150, and 200 μM before mixing) were rapidly mixed at 25 °C in a 1:1 ratio. Heme reduction was followed at 423 nm in a rapid-scanning stopped flow instrument (On-Line Instrument Systems, Inc.).

**Detergent Exchange**—Purified HDR was diluted 4-fold with 25 mM Tris, pH 7.6, 10% glycerol, 0.6% Triton X-100, 2 mM dithiothreitol, and loaded on a DEAE-Sephacel (Sigma) column pre-equilibrated with the same buffer. After washing the column with 25 mM Tris, pH 7.6, 10% glycerol, 2 mM dodecylmaltoside, 2 mM dithiothreitol for at least 5 column volumes, HDR was eluted with 0.4 M KCl-containing dodecylmaltoside buffer. The enzyme was concentrated for freeze-quench EPR experiments.

**Freeze-quench EPR**—Thionin-oxidized HDR (60 μM) was rapidly mixed with 200 μM HPhenH2, at room temperature using a chemical/freeze-quench apparatus (Update Instrument, Inc., Madison, WI). The HDR-containing and HPhenH2-containing syringes (2 ml) were connected by a mixer, and the reaction time was controlled using aging hoses of different lengths. The solutions were mixed using four sequential ram displacements of 1.3 mm (83 μl per shot) and ram velocity of 8 cm/s. The reaction was quenched by rapidly freezing the mixture in a funnel attached to an EPR tube that was filled with low temperature isopentane. The frozen snow containing the quenched reaction mixture was then packed tightly in EPR tubes. The 0-ms time point was obtained by mixing the oxidized HDR with buffer.

**Mersalyl Acid Treatment**—Oxidized HDR (above) was treated with 1 mM mersalyl acid for 30 min. Then, excess mersalyl acid was removed by centrifuging the solution through a Sephadex G-25 column (Amersham Pharmacia Biotech).

**RESULTS**

**Steady State**—Steady-state kinetic experiments were performed at 55 °C at varying concentrations of HPhenH2 and CoB-S-S-CoM to determine the overall mechanism of the HDR reaction. The data were fitted to the Michaelis-Menten equation (Fig. 1), which yielded the $K_m$ values for HPhenH2 and CoB-S-S-CoM of 92 ± 22 and 144 ± 33 μM, respectively. The specific activity was 52 pmol min⁻¹ mg⁻¹ $k_{cat}$ of 70 s⁻¹, assuming a dimeric unit of 80 kDa. In a ternary-complex mechanism, the $V/K$ value for one substrate increases with the concentration of the other substrate. The $V_{max}/K_m$ values for CoB-S-S-CoM do not increase with HPhenH2 concentration (Fig. 1, inset), indicating that the reaction follows a ping-pong mechanism. At higher concentrations of HPhenH2, the $V/K$ value decreases, indicating some degree of substrate inhibition.

**Pre-steady-state Kinetics**—The electron transfer pathway from HPhenH2 to HDR was studied by rapid-scanning stopped-flow kinetics at 25 °C. The enzyme used in the stopped-flow experiments was highly active, with a turnover number at 25 °C of 72 s⁻¹. HPhenH2 was rapidly mixed with oxidized HDR, and heme reduction was monitored at 423 nm. Two phases of equal amplitude were evident, and the data fit well to a biexponential equation, corresponding to reduction of the two b-type hemes of HDR (Fig. 2). Only the first phase of this reaction appears to be kinetically relevant (87 s⁻¹), because the rate constant for the second phase (9.4 s⁻¹) is significantly slower than the turnover number (72 s⁻¹) for the enzyme. Results described below indicate that it is the low potential heme that is reduced at catalytically competent rates. Furthermore, only the first rate constant is dependent on HPhenH2 concentration; the heme reduced in the second phase was HPhen-independent. These results suggest that one heme (the high potential heme, see below) is not involved in CoB-S-S-CoM reduction by HPhenH2.

We had hoped to independently monitor reduction of the heme and the Fe-S cluster; however, the heme absorbance dominated the spectra of HDR. When the HPhenH2 concentration is similar to that of HDR and is well below its $K_m$ value, there are two clearly distinguishable phases: a rapid increase in absorbance ($k_{obs1} = 34 s^{-1}$) followed by a slower decay ($k_{obs2} = 2.3 s^{-1}$) (Fig. 3). The difference spectrum generated by subtracting the spectrum collected at 300 ms from that at 70 ms (Fig. 3B) matches that of the difference spectrum of the reduced minus oxidized HDR. The amplitudes of the two phases are...
equal and correspond to 0.25 heme. The electron acceptor is likely to be either an FeS cluster or some other redox site on the protein, possibly a redox-active disulfide. If the acceptor is an FeS cluster, the results would be most consistent with reoxidation of the heme group by the high-potential Fe-S cluster. Given the slow rate of heme reoxidation, however, this event is unlikely to be involved in catalysis.

**Freeze-quench EPR**—Because we are unable to follow the reduction of FeS clusters by stopped-flow due to dominating absorbance of hemes at 400–450 nm, rapid freeze-quench EPR studies were performed. The FeS cluster is fully reduced within 35 ms (Fig. 4A). The rate constant for Fe-S reduction by HPhen

is $73 \pm 31 \text{s}^{-1}$ at 20 °C (Fig. 4B). These results clearly show that the FeS cluster is reduced at catalytically competent rates.

Given the standard error in the freeze-quench measurement, we cannot conclude whether the cluster is reduced before, after, or simultaneously with the low-potential heme. It is the high-potential cluster that undergoes reduction, because HPhenH$_2$ reduces only the high-potential FeS cluster; the low potential cluster remains oxidized (see below).

The EPR signal of the low potential heme is observed at very low intensity (6) and could not be detected in the freeze-quench
oxidized 415 ms after reaction with HPhenH₂. The rate con-
-ever, even at 1 mM concentration, CO does not affect the rate of
Kₐ 
CO binds tightly to one of the hemes (Fig.
DPI at the following concentrations: 0 ( ), 1 ( ), 2 ( ), and 5 ( ) μM.
The data points were fit globally to the equation describing competitive
The derived kinetic parameters were: Kᵣ for HPhenH₂, 31 μM; Kᵢ for DPI, 0.7 μM; Vₘₐₓ, 38 μmol min⁻¹ mg⁻¹.

Effects of CO on the HDR Reaction—We showed earlier that CO binds tightly to one of the hemes (Kᵣ = 6.2 μM) (6). However, even at 1 mM concentration, CO does not affect the rate of reduction of CoB-S-S-CoM when HPhenH₂ or methyl viologen is the electron donor (Fig. 5). Because only the high spin high potential heme binds CO (6), these results indicate that only the low potential heme is required for CoB-S-S-CoM reduction.

When dithionite is added to HDR, both hemes are reduced (Fig. 6A). When excess CoB-S-S-CoM is then added, the UV-visible spectrum shows a broad Soret peak around 420 nm (Fig. 6B, solid line). This spectrum can be fit to the sum of two hemes with 66% in the oxidized and 34% in the reduced state (dashed line). Therefore, the remaining reduced heme is not involved in substrate reduction. To determine which of the two hemes is involved in catalysis, we added CO to the CoB-S-S-CoM treated enzyme. The Soret peak for the reduced heme shifts to 420 nm (dotted line), which corresponds to the CO-bound form of HDR (6) and the spectrum of the oxidized heme was unchanged. This result clearly shows that, when CoB-S-S-CoM is added, the high potential heme remains reduced and the low potential heme undergoes oxidation.

These combined results strongly indicate that the high potential heme is not involved in CoB-S-S-CoM reduction and that electrons from the reduced low potential heme can reduce CoB-S-S-CoM to the dithiol products at kinetically relevant rates.

Effects of Mersalyl Acid—Mersalyl acid is known to disrupt FeS clusters (20). Addition of mersalyl acid to HDR only slightly affects the heme spectra. This indicates that mercury treatment does not alter the heme environment. This is expected, because mercury only affects hemes with sulfur ligands, not histidine-ligated hemes like those in HDR (6). The difference spectrum between the native and the mersalyl acid-treated HDR showed a broad band around 400–500 nm that is characteristic of FeS clusters (Fig. 7). There is a small peak at 420 nm above the broad absorption band, which is likely to be from the heme. However, this would constitute less than 10% alteration of the heme. Using a typical extinction coefficient for ferredoxin, which is 16 mM⁻¹ cm⁻¹ per cluster (21), these results indicate that the mersalyl acid disrupted 1.8 clusters per dimeric unit. HDR contains two [4Fe-4S] clusters (6).

HPhenH₂ was unable to reduce the hemes of the mersalyl acid-treated oxidized enzyme, whereas dithionite reduced both hemes. The mersalyl acid-treated enzyme was also unable to catalyze the reduction of CoB-S-S-CoM, when either HPhenH₂ or methyl viologen was used as the electron donor. Assuming that mersalyl acid only affects the FeS cluster, these results indicate that an iron-sulfur cluster is the initial acceptor of electrons from HPhenH₂. Another possibility is that disruption of the FeS cluster damages the HPhen binding site, which would prevent heme reduction. The redox potentials of the two clusters are −100 and −400 mV (6). Because the midpoint redox potential for HPhen/HPhenH₂ is −250 mV, it seems likely that the high potential cluster is the electron acceptor from HPhenH₂. Because the results described above indicated that the high potential heme is not involved in CoB-S-S-CoM reduction, we hypothesize that the electron pathway from HPhenH₂ to the heme is: HPhenH₂ → [Fe₄S₄] → [heme b]₉, where the H and L subscripts designate the high and low potential centers. However, this is not the most thermodynamic electron transfer pathway, because the midpoint potential of the low potential heme is 80 mV more negative than that of the high potential cluster.

Effects of Diphenylene Iodonium—Diphenylene iodonium (DPI) is a lipophilic reagent that inhibits a variety of flavoproteins such as NAD(P)H-dependent dehydrogenases and oxidases (22–24). The inhibitor is thought to interact with flavins and low potential b-type cytochromes in these enzymes. DPI also inhibits the reduction of CoB-S-S-CoM by factor F₄₂₅H₂ dehydrogenase in the membrane-bound electron transport chain of M. mazei G61 (25). To elucidate the intramolecular electron transfer pathway among the centers of HDR, we studied inhibition of the purified enzyme by DPI.

Inhibition of the HDR Reaction by DPI—When HPhenH₂ is the electron donor, DPI is a strong inhibitor of CoB-S-S-CoM reduction (Fig. 8A). DPI inhibits heterodisulfide reduction in a competitive manner with respect to HPhenH₂ with a Kᵢ value below 1 μM (Fig. 9). Surprisingly, it increases the rate of methyl viologen oxidation by CoB-S-S-CoM (Fig. 8B). When CoB-S-S-CoM is absent, methyl viologen oxidation is not observed. These results suggest that the mechanism of CoB-S-S-CoM reduction is different with the two electron donors. One possibility is that DPI, in competing with the HPhen binding site,
blocks electron transfer to the high potential Fe-S cluster. However, methyl viologen, which can reduce CoB-S-S-CoM in the presence of DPI, has a different binding site than HPhen; it may transfer electrons directly to the heme.

**UV-visible Spectra of DPI-treated HDR**—The effects of DPI on the oxidation states of the metal centers of HDR are summarized in Table I. When HPhenH₂ is used as the electron donor, both of the hemes are reduced (Fig. 10). Adding DPI to the HPhenH₂-reduced enzyme causes the Soret band at 425 nm to shift to 410 nm, corresponding to the oxidized form of HDR. This indicates that both hemes are oxidized by DPI. Dithionite reduces both hemes in the absence of DPI. However, when dithionite is then added to HPhenH₂-reduced and DPI-oxidized HDR, a composite spectrum is obtained with peaks at 425 and 410 nm. The spectrum fits a mixture of 50% oxidized and 50% reduced heme. These results indicate that, although dithionite has a low enough potential to reduce both hemes, reduction of one of the two hemes is inhibited by prior treatment with DPI. CO shifts the spectrum of the reduced heme in the DPI-treated enzyme indicating that, after treatment of HDR with DPI, only the high potential 5-coordinate heme can be reduced. Reduction of the low potential hexacoordinate heme is inhibited. This constitutes further evidence that the low potential, but not the high potential, heme is involved in electron transfer from HPhenH₂ to the heterodisulfide.

**EPR Spectra of DPI-treated Enzyme**—EPR spectroscopy was used to evaluate the effects of DPI on the metal centers in HDR (Fig. 11). Oxidized HDR displays an EPR spectrum with g values at 6.2, 5.8, and 2.0 that derives from the high spin (spin = 5/2) heme (6) (Fig. 11A). The low spin heme displays a “large g<sub>max</sub>” EPR spectrum with very low intensity and values of g<sub>max</sub> that are >3. When the oxidized enzyme is reduced with HPhenH₂ (Fig. 11B), the high spin heme spectrum disappears and a new EPR signal with g values at 2.06, 1.95, and 1.90 appears, which is from a singly reduced [4Fe-4S] cluster (6). Unlike dithionite (Fig. 11C), HPhenH₂ is not a strong enough electron donor to reduce the low potential cluster. When DPI is added to HPhenH₂-reduced HDR (Fig. 11D), the EPR spectrum of the cluster disappears as the g = 6 signal from the high spin heme reappears. These results combined with the UV-visible spectroscopic results described above indicate that the high potential FeS cluster and the two hemes undergo oxidation by DPI. When the thionin-oxidized enzyme is treated with dithionite (Fig. 11C), all the metal centers of HDR are reduced; the characteristic complex spectrum of the doubly reduced protein is observed with g values at 2.03, 1.97, 1.92, and 1.88 (6). The complicated signals result from dipolar coupling between the two clusters. Addition of DPI to the dithionite-reduced enzyme led to the oxidation of one cluster (Fig. 11E). Presumably, DPI does not oxidize the high potential cluster.

**DISCUSSION**

Electron transfer from HPhenH₂ through the redox centers of HDR to CoB-S-S-CoM drives the translocation of two protons across the cytoplasmic membrane per two electrons transferred (13). Reduction of HDR by HPhenH₂ has been studied here by kinetic and spectroscopic methods.Stopped-flow and freeze-quench EPR experiments indicate that only one of the two hemes and one of the two clusters undergoes reduction by HPhenH₂ with rate constants exceeding k<sub>cat</sub>. Our CO-binding and DPI inhibition experiments indicate that the low potential heme is involved in electron transfer pathway and that the high potential heme is not involved in catalysis. This is consistent with the redox demands of the reaction; i.e. the midpoint potential for the CoB-S-S-CoM/RSH₂ couple is approximately ~200 mV, whereas that of the high spin high potential heme is ~23 mV (6).

Why would HDR retain an unnecessary high potential heme...
Throughout evolution? One possibility is that this heme is involved in stabilizing the protein or in generating the proton gradient associated with the HDR reaction. There is a high potential c-type cytochrome in the membranes of *M. mazei* with unknown function. It is attractive to consider an electron transfer chain leading from the oxidized heme to cytochrome c that would be coupled to proton translocation. Such a pathway would make the HDR reaction analogous to cytochrome bc₁.

Which redox center is the direct electron acceptor from HPhenH₂? HPhenH₂ reduces the FeS cluster and the low potential heme at similar rates. Thus, the rapid kinetics experiments cannot distinguish between the following electron transfer pathways: HPhenH₂ → FeS → heme₃₁, HPhenH₂ → heme₃ → FeS, or a simultaneous reduction of heme₃ and FeS by HPhenH₂. Inhibition experiments provide further information about the possible electron transfer pathways. HPhenH₂ does not reduce either heme group of mersalyl-treated HDR. Because the heme spectra are not appreciably altered by the treatment and mercury is known to disrupt FeS clusters, we propose that inhibition of heme reduction is due to a direct treatment and mercury is known to disrupt FeS clusters, which would in turn donate electrons to the high potential cluster. The electron transport pathway from CODH and CO, with a midpoint potential below ~500 mV, has not been elucidated.

The experiments described in this report were performed in aqueous solution with the water-soluble HPhen analog of MPhen. It is important to determine whether the membrane-bound enzyme uses the same electron transfer pathway. If the physiologically relevant order of electron flow is indeed HPhenH₂ → [Fe₄S₄]H → [heme b₂], the high potential cluster is expected to be located near the subunit interface. This is because the large subunit containing the clusters is cytoplasmic and the heme is in the membrane-associated subunit (16). This would be similar to several quinone-coupled enzymes whose FeS clusters are in close contact with a quinone-binding site in membrane, such as quinol:quinone reductase (26), Me₃S⁻ reductase (27), and NADH:ubiquinone oxidoreductase (28).

How do electrons passing through one-electron redox centers accomplish the two-electron reduction of CoB-S-S-CoM? Because the two classes of HDRs are heme iron-sulfur or flavin iron-sulfur proteins, Thauer et al. (16) proposed that the iron-sulfur subunits harbor the active site of heterodisulfide reduction and that the mechanism of disulfide reductase could resemble that of the ferredoxin:thioredoxin reductases from chloroplasts and cyanobacteria. In the plant thioredoxin reductase mechanism, a sulfur radical, which is formed as an intermediate, appears to be stabilized by binding to a [Fe₄S₄] cluster (29, 30). Evidence presented here indicates that the low potential cluster of HDR is not involved in electron transfer reactions. Because the midpoint potential of this cluster (−400 mV) is much lower than that of HPhen (−250 mV) or CoB-S-S-CoM (−220 mV), this cluster may play a catalytic role, analogous to that of the cluster in thioredoxin reductase. This putative role is to stabilize a radical anion formed by one electron reduction of the disulfide substrate. Further studies are required to test this hypothesis.

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