Characterization of membrane-bound sulfane reductase: A missing link in the evolution of modern day respiratory complexes

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Hyperthermophilic archaea contain a hydrogen gas–evolving, respiratory membrane-bound NiFe-hydrogenase (MBH) that is very closely related to the aerobic respiratory complex I. During growth on elemental sulfur (S°), these microorganisms also produce a homologous membrane-bound complex (MBX), which generates H2S. MBX evolutionarily links MBH to complex I, but its catalytic function is unknown. Herein, we show that MBX reduces the sulfane sulfur of polysulfides by using ferredoxin (Fd) as the electron donor, and we name it membrane-bound sulfane reductase (MBS). Two forms of affinity-tagged MBS were purified from genetically engineered *Pyrococcus furiosus* (a hyperthermophilic archaea species): the 13-subunit holozyme (S-MBS) and a cytoplasmic 4-subunit catalytic subcomplex (C-MBS). S-MBS and C-MBS reduced dimethyl trisulfide (DMTS) with comparable *Km* (~490 μM) and *Vmax* values (12 μmol/min/mg). The MBS catalytic subunit (MbsL), but not that of complex I (NuoD), retains two of four NiFe-coordinating cysteine residues of MBH. However, these cysteine residues were not involved in MBs catalysis because a mutant *P. furiosus* strain (MbsLC85A/C385A) grew normally with S°. The products of the DMTS reduction and properties of polysulfides indicated that in the physiological reaction, MBS uses Fd (E′ < ~480 mV) to reduce sulfane sulfur (E′ < ~260 mV) and cleave organic (RS2R, n ≳ 3) and anionic polysulfides (S2−, n ≳ 4) but that it does not produce H2S. Based on homology to MBH, MBS also creates an ion gradient for ATP synthesis. This work establishes the electrochemical reaction catalyzed by MBS that is intermediate in the evolution from proton- to quinone-reducing respiratory complexes.

The very close evolutionary relationship between respiratory membrane-bound NiFe-hydrogenases (MBH) found in anaerobic microbes and complex I of the mitochondrial aerobic respiratory chain is well-established (1–3). For example, the MBH of *Pyrococcus furiosus* oxidizes reduced ferredoxin (Fd) produced by fermentation, reduces protons to H2 gas, and conserves energy by creating a sodium ion gradient that is used by ATP synthase to form ATP (4). MBH is encoded by a 14-gene operon, and virtually all of its subunits have close homologs in complex I (Fig. 1). Indeed, the recent cryo-EM structure of MBH showed that its H2-evolving, electron transfer cytoplasmic module and ion-pumping membrane module are virtually superimposable on components of complex I despite different overall architectures (5). The cytoplasmic module common to MBH and complex I contains three [4Fe-4S] clusters that channel electrons (from the oxidation of Fd or NADH) to the catalytic subunit (MbhL or NuoD), and this reduces either protons or a quinone derivative (menaquinone or ubiquinone), respectively. Elucidating the evolutionary relationship between the quinone-reducing site of complex I of the aerobic respiratory chain and the proton-reducing NiFe site present in MBH is therefore a fundamental issue.

The catalytic subunits of NiFe-hydrogenases fall into four phylogenetic groups, three of which (groups 1–3) are clustered together along with a distantly related group 4 (2, 6). Only the group 4 hydrogenases include nonhydrogenase catalytic subunits. In addition to that (NuoD) of complex I, they are FpO of a methanophenazine-reducing respiratory complex (F420H2:phenazine oxidoreductase, FPO) found in methanogens (7, 8) and MbxL of a membrane-bound respiratory complex (MBX) of unknown function that is produced by *P. furiosus* and related organisms during growth with elemental sulfur (5) (9). It has been proposed (2) that MBH, MBX, FPO, and complex I evolved from a common H2-evolving ancestor and that this was driven by the reduction of oxidants with increasing electrochemical potential. For example, the free energy released in coupling the oxidation of ferredoxin (E′ < ~480 mV) to the reduction of protons, S°, phenazine, and ubiquinone is 12, 41, 61, and 112 kJ/mol, respectively (2). Such terminal electron acceptors likely became available as the Earth’s atmosphere and oceans became more and more oxidized. The first step in the eventual evolution of a quinone-reducing complex may have been the appearance of S° in the Archean (10, 11), and this drove the evolution of a S°-reducing respiratory system based on MBH. Characterization of MBX is therefore obviously required.
Characterization of MBS from *P. furiosus*

Figure 1. Diagrammatic representations of the MBH and the membrane-bound oxidoreductase of unknown function (MBX) of *P. furiosus* and of complex I. Homologous subunits are in the same color. The number of cysteiny1 residues in the red catalytic subunits are indicated by -SH groups. (The figure was modified from Ref. 2.)

to understand this evolutionary transition at the molecular level. The goal of the present work was to purify the MBH-related MBX system from *P. furiosus* and to determine its role in S\(^\bullet\) reduction.

MBX is ubiquitous within the order of the S\(^\bullet\)-reducing Thermococcales. It is believed to be the product of an operon duplication of a proton-reducing MBH-like respiratory system that allowed the switch from proton to S\(^\bullet\) reduction (2, 12, 13). *P. furiosus* MBX is encoded by a 13-gene operon and contains homologs of all of the 14 subunits of *P. furiosus* MBH, except that of MbhI, as shown in Fig. 1. Hence MBX retains the same four subunit electron transfer module containing three [4Fe-4S] clusters. Based on the high homology with MBH, MBX was also proposed to retain the ion translocation channels in the membrane-bound subunits (2). One major difference between MBH and MBX is that the proposed catalytic subunit, MbxL, lacks two of four cysteine residues that coordinate the [NiFe] active site in MbhL. The same is true for the catalytic subunit (FpoD) of FPO, whereas that of complex I (NuoD) lacks all four of these cysteine residues (Fig. 1). The great similarity between MBX and MBH is indicated by the fact that the mbx operon was originally proposed to encode a NiFe hydrogenase with unusual ligand coordination (14). However, the MBX deletion strain (∆mbxl) of *P. furiosus* grows very poorly on S\(^\bullet\), showing that MBX is essential for efficient S\(^\bullet\) respiration (9).

*P. furiosus* grows optimally at 100 °C using carbohydrates or peptides as carbon sources to produce acetate, CO\(_2\), and H\(_2\) in the absence of S\(^\bullet\) (15). Adding S\(^\bullet\) to a growing culture of *P. furiosus* stops H\(_2\) production, and H\(_2\)S is produced instead (16). Within 10 min of S\(^\bullet\) addition, no Fd-dependent NADP\(^\bullet\) oxidation was found (9, 16). This putative NSR–MBX type of S\(^\bullet\)-reduction activity could be measured in cell extracts was NADPH-dependent (16). The enzyme responsible, NADPH sulfur reductase (NSR), was characterized, and its expression was also regulated by SurR. It was therefore proposed that MBX oxidizes Fd and reduces NADP\(^\bullet\), which is then used by NSR to reduce S\(^\bullet\) (9, 16). This putative NSR–MBX type of S\(^\bullet\)-reduction activity could be measured in the cell extracts or membranes from *P. furiosus*. The reaction catalyzed by MBX and its physiological function were therefore unresolved (9). Herein, we report the purification and characterization of MBX and the identification of a sulfane sulfur substrate. We also show that MBX is unlikely to produce H\(_2\)S directly and that the two conserved cysteine residues in the MbxL catalytic subunit are not involved in catalysis. The results presented here provide new insights into this hitherto mysterious membrane-bound complex in terms of physiological function and its evolutionary relationship to MBH and complex I.

Results

The MBX operon and construction of affinity-tagged MBX

The 13-gene operon encoding MBX is annotated based on homology with the genes encoding MBH (Fig. 2). MBX lacks an MbhI homolog but contains a duplication of MbhH (MbxH\(^\bullet\)) and a fusion of MbhE and MbhF (MbxE). Hence, MBX is encoded by MbxA-EGH′MJKLN. The last four genes in the operon, MbxJKLN, encode the cytoplasmic module and contain the active site (MbxL) and iron–sulfur clusters, whereas the first nine genes all encode subunits with multiple transmembrane helices and form the membrane arm (Fig. 1).
Our previous attempts to find a substrate for MBX using *P. furiosus* membranes had been plagued by nonspecific reactions because of the high reactivity of potential sulfur-containing substrates, such as S\(^{-}\) and polysulfides (see below), and the presence of S\(^{-}\)-derived compounds in the membranes of S\(^{-}\)-grown cells (even after extensive washing). The goal here was therefore to obtain the solubilized holoenzyme form of MBX to remove possible interferences from other membrane components and also to obtain the much simpler cytoplasmic subcomplex to minimize possible side reactions. The strategy to do this is shown in Fig. 2. The 13-gene mbx operon was split into two transcripts by placing the transformation marker and a highly active constitutive promoter, P\(_{slp}\), which controls expression of the gene encoding the S-layer protein, upstream of mbx\(_J\). The construct also included a His\(_9\) tag at the N terminus of MbxJ. These two new operons should result in two transcripts controlled by two promoters: mbxA-M under the control of the native mbx promoter and mbxJ-N driven by P\(_{slp}\). Based on qPCR analysis (Fig. S1), the copy number of RNA molecules encoding mbxJ-N was six times higher than that encoding mbxA-M. Assuming the transcripts are stable and translated into proteins with similar efficiencies, we anticipated that two versions of MBX would be produced, 9-subunit MbxA-M and 4-subunit MbxJ-N with an affinity tag at the N terminus of MbxJ. MbxA-M should combine with MbxJ-N to give the intact 13-subunit holoenzyme (MbxA-N) in the membrane, whereas excess 4-subunit MbxJ-N should accumulate in the cytoplasm.

**Purification of S-MBX and C-MBX**

For the purification of both forms of MBX, all steps and procedures were carried out under strict anaerobic conditions. The affinity-tagged holoenzyme form of MBX (MbxA-N) was solubilized from washed *P. furiosus* membranes by incubation with 10% (v/v) Triton X-100 for 16 h at 4 °C (the same procedure used to solubilize MBH (23)) and purified by two steps of affinity chromatography. The solubilized and purified holoenzyme, now designated S-MBX (MbxA-N), was analyzed by SDS–PAGE. All 13 subunits could be assigned based on their predicted size (Fig. 3) and all 13 were identified by LC–MS/MS (LC–MS/MS) analysis. To purify the affinity-tagged 4-subunit subcomplex from the cytoplasm, the supernatant fraction from the membrane preparation was concentrated by anion-exchange chromatography and then subjected to affinity chromatography. When the purified fraction was analyzed by SDS–PAGE (Fig. 3), all four subunits (MbxJ-N) were evident based on their expected sizes and each was identified by LC–MS/MS and MALDI–TOF. This cytoplasmic subcomplex is termed C-MBX. The sizes of the two purified complexes estimated by gel filtration chromatography were 1.2 MDa for S-MBX (calculated to be 357 kDa for MbxA-N) and 125 kDa for C-MBX (calculated to be 113 kDa for MbxJKLN). These data indicate that the S-MBX holoenzyme is purified as a trimeric complex, whereas C-MBX is a monomeric form of the heterotetramer. By inductively coupled plasma–mass spectrometry (ICP–MS)
analysis (for iron, magnesium, cobalt, molybdenum, vanadium, nickel, manganese, zinc, tungsten, and lead), iron was the only metal detected in the two enzyme forms, thereby confirming the absence of nickel and molybdenum and supporting the model shown in Fig. 1.

With highly purified holoenzyme S-MBX and cytoplasmic subcomplex C-MBX in hand, we investigated their ability to use titanium (III)-reduced *P. furiosus* Fd to reduce S° directly, where the oxidation of Fd was measured to monitor activity. However, no significant activity was detected above the background over the temperature range 60–80 °C for the direct reduction of S° by reduced Fd. All artificial electron donors that were used (reduced by sodium dithionite) in place of Fd, including methyl viologen (MV), benzyl viologen, phenoasafranine, safranin O, and thionine, also reacted very rapidly with S°, and the addition of S-MBX or C-MBX did not have any effect. Although NADH and NADPH did not react directly with S°, neither MBX complex catalyzed their oxidation. Attempts to replace S° with polysulfide and sodium tetrasulfide were also unsuccessful because no activity could be measured above the background. In contrast, dimethyl disulfide (CH₃S₂CH₃) and dibenzyl trisulfide (PhCH₂S₃CH₂Ph) did not react with titanium (III)-reduced MV at an appreciable rate at 80 °C, but no significant stimulation of activity was found when either S-MBX or C-MBX were added. However, the reduction of dimethyl trisulfide (CH₃S₃CH₃, DMTS), which also gave only a low background activity with reduced MV, was dramatically enhanced by the addition of either S-MBX or C-MBX. DMTS is stable at 80 °C (6% decomposition after 20 h (24)) and was therefore used as the model substrate for MBX.

**Biochemical properties of S-MBX and C-MBX**

A standard in vitro assay for MBX was developed using DMTS (2.0 mM) as the electron acceptor and titanium-reduced MV as the electron donor. With this assay we were unable to measure significant activity using washed *P. furiosus* membranes because of the background interference (compared with cells grown in the absence of S°) arising from the S° and S°-derived compounds within the membranes of S°-grown cells. Once these interferences were removed from the membranes by detergent treatment, DMTS reduction activity was measurable, and this allowed S-MBHH purification to be followed by activity. The results are summarized in Table 1. S-MBX was purified 110-fold with 23% recovery of activity and a yield of 3.6 mg from 100 g (wet weight of cells). The purified enzyme had a specific activity of 9.9 units/mg. As shown in Table 2, C-MBX, was purified 33-fold with a yield of 4.2 mg from 100 g (wet weight of cells). The purified enzyme had a specific activity of 9.5 units/mg. However, the recovery of activity of C-MBX was only 2%, suggesting that some other cytoplasmic enzymes are able to reduce DMTS. This was not too surprising because S° is reduced to sulfide nonspecifically at 80 °C by several *P. furiosus* oxidoreductases, including hydrogenase (SHI), NADH-dependent ferredoxin NAD oxidoreductase (Nfn) and pyruvate ferredoxin oxidoreductase (25, 26). Analysis by MS showed that MBX reduced DMTS using methyl viologen as the electron donor to produce both methanethiol (CH₃SH) and methyl hydrogen disulfide (CH₃S-SH; Fig. S2) but H₂S could not be detected as a product by the standard colorimetric assay.

As shown in Fig. 1, MBX is proposed to use reduced Fd as an electron donor in vivo, and this was confirmed by the in vitro assay. Titanium-reduced Fd was able to replace titanium-reduced MV with both S-MBX and C-MBX with specific activities 3.8 and 3.2 units/mg, respectively. This suggests that the
iron–sulfur clusters responsible for the electron transport from MbxA to MbxB were intact in the purified enzymes, as shown Fig. 1. The kinetic properties of S-MBX and C-MBX are summarized in Table 3. According to the $K_m$ values, S-MBX has a slightly higher affinity for DMTS than does C-MBX, and the low value ($\sim 0.4$ mM) is consistent with the use of DMTS as a model sulfur-containing substrate. The $V_{max}$ values were comparable for the two enzymes ($\sim 12$ units/mg). Because the molecular weight of C-MBX is approximately one-third of that of S-MBX, this suggests that C-MBX is not as active as it is when part of the holoenzyme complex. The lower-than-expected activity of C-MBX is not due to the stability of the enzyme because, surprisingly, C-MBX was more thermostable than S-MBX. The loss of activity at 90 °C was a first-order reaction in both cases with half-lives of 28 and 7 h, respectively. Accordingly, S-MBX and C-MBX exhibited similar sensitivities to inactivation by oxygen with half-lives in air (at 25 °C) of 19 and 17 h, respectively. The optimal temperature for DMTS reduction by both S-MBX and C-MBX was above 90 °C with almost an order of magnitude increase in activity upon increasing the assay temperature from 60° to 90 °C (Fig. S3).

### The role of the cysteine residues in MbxL

As shown in Fig. 1, the catalytic subunit, MbhL, of MBH contains four cysteine residues that coordinate the catalytic NiFe site, and two of these are conserved in MbxL (alignments are given in Fig. S4). Hence, a key question is whether these two residues are essential for catalysis by MBX. We constructed a strain of *P. furiosus* wherein the MbxL subunit of MBX contained a double mutation, C85A and C385A. We had previously shown that a mutant strain of *P. furiosus* (ΔMbxL) lacking the gene encoding MbxL grew very poorly in the presence of S°, and as anticipated, the MbxL-C85A/C385A strain showed no growth defect on S° and grew the same as the MbxL-WT strain (9). We therefore constructed two strains based on the ΔMbxL strain. One (MbxL-WT) contained the WT *mbxL* gene, whereas the other (MbxL-C85A/C385A) contained *mbxL* with the double mutation, C85A and C385A. Both genes were inserted as part of a mbxJKLN construct into the intergenic space between PF0265 and PF0266 (an intergenic space with no detectable transcriptional activity; data not shown). As shown in Fig. 4A, the ΔMbxL strain has minimal growth on S°, and as anticipated, the WT *mbxL* complemented the growth defect. However, and somewhat surprisingly, the MbxL-C85A/C385A strain showed no growth defect on S° and grew the same as the MbxL-WT strain. Moreover, as shown in Fig. 4B, the MbxL-WT and MbxL-C85A/C385A strains produced comparable amounts of H$_2$S, and the rates of production matched the growth rates of both strains. Hence, the defect in S° metabolism caused by the deletion of MbxL was restored by the MbxL subunit even when the two cysteine residues conserved in MBX were replaced by alanine residues. In addition, these results show that these two cysteine residues do not coordinate a unique type of iron center in MbxL that could be important for catalytic activity, although we cannot rule out the presence of a novel iron site coordinated by noncysteiny1 residues.

### Discussion

Herein we describe the purification and characterization of the solubilized holoenzyme S-MBX and the cytoplasmic subcomplex C-MBX of *P. furiosus*. The two subcomplexes of the holoenzyme, MbxA-M and MbxB-N, are encoded by separate transcripts and translated independently (Fig. 2), yet they combine to generate a functional MBX, which is essential for cells to grow in the presence of S°. In the recombinant strain, the SurR-regulated promoter of *mbx* (27) controls production of the MbxA-M subcomplex such that it is only produced in S°-grown cells, but the generation of the MbxB-N subcomplex is driven by the constitutive promoter, P$_{kol}$. Thus, the cytoplasmic subcomplex C-MBX should be produced at comparable levels in cells grown both with and without S°. However, we were unable to purify C-MBX by affinity chromatography of the cytoplasmic extract of cells grown without S°. This suggests that accessory
proteins whose genes are expressed only in the presence of $S^\circ$ might be required for production of a stable MbX-N subcomplex, although what those might be is not known at present. On the other hand, the C-MBX subcomplex (MbxJ-N) produced in $S^\circ$-grown cells could be purified and was extremely stable; in fact, it was more stable at 90 °C than the purified holoenzyme (S-MBX). This might be due to the presence of Triton X-100 used to solubilize S-MBX because the cloud point is $\sim 70$ °C, above which micelles would aggregate to form a detergent-rich phase that could destabilize the holoenzyme (28).

Past attempts to assign a catalytic activity to the MBX complex in vitro using P. furiosus membranes suffered from high background interference because potential substrates are highly reactive sulfur species ($S^\circ$ and polysulfide, see below) that directly oxidize potential electron donors and do so completely in seconds, particularly at high temperatures (80 °C). This situation is compounded by the apparent presence of $S^\circ$ and $S^\circ$-derivatives in the membranes of $S^\circ$-grown cells, even after extensive washing, that also react directly with potential electron donors (this reactivity is not evident with membranes from cells grown without $S^\circ$). These problems were overcome by affinity tagging and purifying MBX and by the use of several sulfur-containing substrates that have minimal reactivity with potential donors. One of these, DMTS ($\text{CH}_3\text{S}_3\text{CH}_3$), was a very effective substrate for MBX. DMTS is a well-studied compound because it is produced by methionine degradation in various bacteria and gives flavor to a variety of foods and beverages (29, 30). However, it is not thought to be a physiological substrate for MBX; rather, it contains a stable three-sulfur polysulfide-like structure that mimics the natural substrate.

Elemental sulfur ($S^\circ$) is also not thought to be a physiological substrate for P. furiosus or any other $S^\circ$-reducing microbe because of its extremely low solubility. Its $S_8$ ring readily reacts with $\text{H}_2\text{S}$ ($\text{HS}^-$, $pK$ 7.0) to generate soluble polysulfides ($S_n^\circ$). In hydrothermal vent environments of the type inhabited by P. furiosus, polysulfides generated by the reaction of geothermally produced $\text{H}_2\text{S}$ with $S^\circ$ are likely to be the true substrates for $S^\circ$-reducing hyperthermophiles (the solubility of polysulfides increases 10-fold from 37 to 90 °C) (31, 32). Above pH 6.0, the reaction of $\text{H}_2\text{S}$ with $S^\circ$ generates predominantly linear tetra- and pentapolysulfides mainly in the diionic $S_n^\circ$ form (their respective $pK$ values are 6.3 and 5.7 (32)). Heptas-, octas-, and nonasulfides are also produced but di- and trisulfides are unstable and readily disproportionate to $S^\circ$ and $\text{HS}^-$ (33, 34). In addition, a variety of organic polysulfides were identified in the cytoplasm of several $S^\circ$-grown hyperthermophilic species, and they are thought to be generated by the reaction of anionic polysulfides with aldehydes produced during amino acid metabolism (35).

The ability of MBX to catalyze DMTS reduction provides insight into its physiological reaction. Methanethiol and methyl hydrogen disulfide are produced when DMTS is reduced by two electrons (Equation 1) but reduction by four electrons produces methanethiol and $\text{H}_2\text{S}$ (Equation 2).

$$\text{CH}_3\text{-S-S-CH}_3 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{CH}_3\text{SH} + \text{CH}_3\text{-S-SH} \quad \text{(Eq. 1)}$$

$$\text{H}_2\text{S} + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{CH}_3\text{SH} + \text{H}_2\text{S} \quad \text{(Eq. 2)}$$

In the MBX assay, $\text{H}_2\text{S}$ was not detected as a product of DMTS reduction, but both $\text{CH}_3\text{SH}$ and $\text{CH}_3\text{-S-SH}$ were detected by MS analysis so MBX catalyzes only the two-electron reduction of DMTS (Equation 1). In addition, MBX did not use dimethyl disulfide or dibenzyl trisulfide as substrates. We therefore conclude that MBX reduces the sulfur–sulfur bond within a trisulfide ($\text{S-S-S}$-) and catalyzes the reduction of organic polysulfides according to the general reaction shown in Equation 3, where $n \geq 3$ and $R$ and $R'$ are alkyl but not aryl. The corresponding reaction with anionic polysulfides ($R = R' = H$) is shown in Equation 4 (where $n \geq 4$ because of the stability of anionic polysulfides).

$$R\text{-S}_x\text{-R}^- + 2\text{e}^- \rightarrow R\text{S}_n^- \text{-x} + R'S_x^- \quad \text{(Eq. 3)}$$

$$S_n^\circ + 2\text{e}^- \rightarrow S_n^- \text{-x} + S_2^- \quad \text{(Eq. 4)}$$

$$S_n^\circ + 2\text{e}^- + 2\text{H}^+ \rightarrow S_{n-1}^- + \text{H}_2\text{S} \quad \text{(Eq. 5)}$$

$$R\text{S}_n^- + 2\text{e}^- + 2\text{H}^+ \rightarrow R\text{S}_{n-1}^- + \text{H}_2\text{S} \quad \text{(Eq. 6)}$$

Any di- and trisulfides that are produced by MBX near neutral pH according to Equation 4 will spontaneously convert to $S^\circ$ and $\text{H}_2\text{S}$. It seems unlikely that MBX generates $\text{H}_2\text{S}$ directly from the reduction of anionic polysulfides (Equation 5) or of monoalkyl polysulfides (Equation 6) generated from Equation 4. Indeed, Equations 5 and 6 represent fundamentally different reactions from those of Equations 3 and 4, which involve simple $2\text{e}^-$ transfer without the need for protons, consistent with the lack in MBX of the proton path to the catalytic site found in MBH (see below). We therefore propose that MBX reduces both organic ($n \geq 3$) and anionic polysulfides ($n \geq 4$) but that it does not generate $\text{H}_2\text{S}$ per se; rather, it produces smaller polysulfides ($S_n^- \text{-x}$), and when $n \leq 3$ they spontaneously disproportionate to $\text{H}_2\text{S}$ and $S^\circ$. Hence, we rename MBX to MB for membrane-bound sulfane reductase.

As shown in Fig. 1, the catalytic subunit (MbxL, now MbsL) of MB contains two cysteinyI residues, and they correspond to the two of the four that coordinate the NiFe catalytic site in MbhL of MBH. However, the growth phenotype of the P. furiosus C85A/C385A mutant strain described herein unequivocally demonstrates that these two cysteine residues are not required for the physiological function of MB. This is consistent with the proposed physiological reaction involving a $2\text{e}^-$ transfer (Equations 3 and 4), in which there is no obvious requirement for an active site thiol. Indeed, the residues of the proposed proton transfer pathway from the cytoplasm to the catalytic site in MBH (5) are not conserved in MBS (Fig. S4), which also supports MBs catalyzing the proton-independent reactions shown in Equations 3 and 4. These two cysteinyI residues in MBS are also conserved in the catalytic subunit (FpoD) of FP0, which catalyzes the reduction of the hydrophobic aromatic phenazine, a reaction that also does not require thiol/disulfide chemistry (2). The same is true for the reaction (quinone reduction) catalyzed by complex I, but in this case the catalytic subunit (NuOD) lacks all four of the NiFe-coordinating cysteine residues of MBH. It therefore appears that although these two
cysteinyl residues in MbsL and FpoD, subunits that are thought to be intermediates in the evolution of NuoD (Fig. 1 and Ref. 2), are conserved, this is not because of catalytic constraints. The reason for their presence is still not clear because they are also not involved in the assembly of MBS because a fully functional complex was produced by the P. furiosus C85A/C385A mutant strain.

Based on the results presented here, we propose that MBS is the primary enzyme responsible for the reduction of polysulfides derived from S° during the growth of P. furiosus. This is consistent with a previous mutational analysis (9) showing that MBS (MBX) is essential for growth on S° but that NSR and two so-called sulfide dehydrogenases (PF1327–1328 and PF1910–1911, now known as Nfnl and NfnII (36)) are not. Similarly, the two sulfhydrogenases of P. furiosus (now known as soluble hydrogenases I and II or SHI and SHII (37, 38)), which can use H₂ or NAD(P)H to reduce S° in vitro, are also not involved in S° reduction in vivo (39). We show here that MBS uses reduced P. furiosus Fd to reduce the model substrate DMTS. MBS is proposed to use the reduced Fd (E°' = 480 mV) produced by glycolysis to reduce organic and anionic polysulfides (Equations 3 and 4; E°' = -260 mV (32)) to generate small anionic polysulfides (n ≤ 3) that spontaneously generate H₂S and S° (Fig. 5). It has been shown that P. furiosus uses polysulfides as growth substrates (40), although exactly how these enter the cell is not clear. By analogy with MBH (Fig. 1), where the ion translocation channels were identified in the MBH structure (5), MBS is also proposed to couple its catalytic reaction to the formation of a net sodium ion gradient, which is used for ATP synthesis (Fig. 5).

There are, however, several fundamental differences between MBS and MBH in addition to the lack of a proton path to the proposed catalytic site in MBS (Fig. S4). These include the lack of a homolog of MbhI in MBX, the duplication of MbhH (MbsH and MbsH') and a fusion of MbhE and MbhF (MbsE) in MBX, and the absence in MbsM of the string of Glu residues (H11032/H11005/H11011/H11002) and a fusion of MbhE and MbhF (MbsE) in MbsM (Fig. S5). In addition, MBX appears to conserve much more energy than MBH (41 versus 12 kJ/mol (2)), leading to a cellular yield coefficient almost twice that obtained in the absence of S° (20). With the advent of a catalytic activity for MBS (Equations 3 and 4) and of a substrate (DMTS) for in vitro assays, it will now be possible to design and characterize MBS mutants based on the MBH structure (5). This will allow us to investigate how these structural differences affect the functions of these two ancestral respiratory complexes from P. furiosus and how they differ in converting an electrochemical potential into an ion gradient.

**Experimental procedures**

**Strain construction**

The strains that were used in this study are summarized in Table S1. The genetically tractable P. furiosus strain COM1 was used for the genetic manipulation of MBX (41). An insertion cassette was constructed using overlapping PCR (42). The upstream flanking region, which also contained the promoter of the gene encoding the S-layer protein (P_slp), and the downstream flanking region were amplified from P. furiosus genomic DNA, and the selection marker (P_gal::pyrF), was amplified from pGLW021 (41). A His tag was also placed at the N terminus of mbxJ (Fig. S6). The split operon after transformation is shown in Fig. 2. Strain MW0572 (MbxL-C85A/C385A) was constructed as shown in Fig. S7. Mutagenesis was carried out using QuickChange II site-directed mutagenesis kit (Agilent Technologies), and the fragments were assembled using overlapping PCR. The insertion cassette was transformed into the intergenic space between PF0265 and PF0266. The transformants were grown as previously described (41), and the PCR confirmed colonies were sequence verified using the Genewiz service.

**Growth conditions**

The growth medium (41) contained 1× base salt, 1× trace minerals, 10 μM tungstic acid, 5 g/liter yeast extract, 5 g/liter casein hydrolysate, 5 g/liter maltose, 0.5 g/liter cysteine, 0.5 g/liter sodium sulfide, 1 g/liter sodium bicarbonate, 1 mM potassium phosphate (pH 6.8), and 5 g/liter S°. The stock solutions of base salt and trace mineral were prepared as previously described (43). All cultures were routinely grown at 90 °C in 100-ml sealed bottles with shaking at 150 rpm. The same medium was used for the 20-liter fermentations that were performed as previously described (44), and the harvested cells were frozen in liquid nitrogen and stored at −80 °C. To monitor growth, 1-ml samples were taken and centrifuged at 14,000 × g for 5 min. The cell pellet was lysed osmotically using 1 ml of distilled H₂O, and cell debris was removed by centrifugation at 14,000 × g for 1 min. The supernatant was collected for protein estimation using protein assay reagent (Bio-Rad). H₂S production in the head-space was analyzed by the methylene blue method (45), and abiotic H₂S production was subtracted from the experimental samples using the control without the addition of cells.

**RNA extraction and quantitative RT–PCR analysis**

MW0491 (MbxJ-His) cells were harvested at mid-log phase, and RNA was isolated using Absolutely RNA miniprep kit (Agilent Technologies). Genomic DNA was digested before cDNA synthesis using TURBO DNase (Ambion), and RNA was purified by a phenol chloroform extraction method as previously described (46). cDNA synthesis was carried out with 1 μg of purified RNA using AffinityScript cDNA synthesis kit (Agilent Technologies), and qPCR analysis was performed using Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies).
Characterization of MBS from *P. furiosus*

with primers designed to amplify ~100-bp products within the target genes: *mbxA* (PF1453), *mbxB* (PF1449), *mbxM* (PF1445), *mbxI* (PF1444), *mbxL* (PF1442), *mbxN* (PF1441), and *pory* (PF0971), where PF0971 was used as the reference gene.

**Purification of S-MBX and C-MBX**

All purification procedures were performed under anaerobic conditions. Frozen cells were lysed osmotically in 25 mM Tris- HCl, pH 8.0, containing 1 mM DTT, and 50 µg/ml DNase I (5 ml/g of frozen cells) for 1 h at room temperature in an anaerobic chamber (Coy Laboratory Products). The cell-free extract was centrifuged at 5,000 × g for 20 s to remove the majority of the S° and then centrifuged at 100,000 × g for 1 h using a Beckman Coulter Optima L-90K ultracentrifuge. The supernatant representing the cytoplasmic extract was used to purify C-MBX. It was applied to a UNOsphere Q column (Bio-Rad) equilibrated with 25 mM Tris- HCl, pH 8.0, containing 1 mM DTT (buffer A) while diluting it 10-fold with buffer A using a NGC chromatography system (Bio-Rad). The bound protein was eluted with buffer B (buffer A containing 0.5 M NaCl). The eluted protein was applied to a 5-ml His-Trap FF Ni–NTA column (GE Healthcare) while diluting it 10-fold with buffer A using a NGC chromatography system (Bio-Rad). The bound protein was block eluted while diluting it 5-fold with 25 mM sodium phosphate, pH 7.5, containing 300 mM NaCl and 1 mM DTT (buffer C). The column was equilibrated with buffer C before loading the sample. The column was washed with five column volumes of buffer C, and the bound protein was eluted with a 20-column volume gradient from 0 to 100% buffer D (buffer C containing 500 mM imidazole). The active enzyme as measured by the MV-linked DMTS reduction assay was pooled and applied to a 5-ml Q HP column (GE Healthcare) while diluting it 10-fold with buffer A. A 20-column volume gradient from 0 to 100% buffer B was used to elute the bound C-MBX.

For the purification of S-MBX, the pellet after 100,000 × g centrifugation step representing the membrane fraction was washed with 50 mM EPPS, pH 8.0, containing 5 mM MgCl₂, 50 mM NaCl, 10% (v/v) glycerol, 2 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. The washed membranes were collected by centrifugation at 100,000 × g for 1 h. This washing step was repeated twice, and the membrane-containing pellet was resuspended in 50 mM Tris- HCl, pH 8.0, containing 0.5 mM MgCl₂, 50 mM NaCl, 5% (v/v) glycerol, 2 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride using a Pyrex tissue grinder (Pyrex). The resuspended membranes were solubilized by stirring with 10% (v/v) Triton X-100 at 4 °C for 16 h. The solubilized membranes were centrifuged at 100,000 × g for 1 h, and the supernatant was collected and applied to a 5-ml His-Trap FF column while diluting it 10-fold with 25 mM sodium phosphate, pH 7.5, containing 300 mM NaCl, 1 mM DTT, and 0.02% (v/v) Triton X-100 (buffer A). The column was equilibrated with buffer A before loading the sample. The bound protein was eluted with a 20-column volume gradient from 0 to 100% buffer B (buffer A containing 500 mM imidazole). The active enzyme as measured by the MV-linked DMTS reduction assay was collected and applied to a 1-ml His-Trap HP column equilibrated with buffer A while diluting it 5-fold with buffer A. A 10-column volume gradient from 0 to 100% buffer B was carried out to elute the bound S-MBX.

The size of S-MBX was estimated using a Superose 6 column (GE Healthcare) that was equilibrated with 50 mM Tris- HCl, pH 8.2, containing 300 mM NaCl, 1 mM sodium dithionite, and 0.02% (v/v) Triton X-100. The size of C-MBX was determined using an ENrich SEC 650 column (Bio-Rad), which was equilibrated with 50 mM Tris, pH 8.2, containing 300 mM NaCl, and 1 mM sodium dithionite. The eluted protein was analyzed by SDS–PAGE using precast Criterion TGX (4–15%) gels (Bio-Rad) and 4–12% Bis-Tris Novex NuPAGE gels (Invitrogen).

**Enzyme assays**

All assays were carried out at 80 °C using anaerobic sealed cuvettes. The standard DMTS reduction assay used a 2-ml reaction mixture containing 100 mM MOPS, pH 7.5, and 150 mM NaCl. After preheating to 80 °C, 1 mM methyl viologen reduced by titanium citrate and 2 mM DMTS were added, and the reaction was initiated by addition of the enzyme. The activity was measured by monitoring the oxidation of methyl viologen at 600 nm (ε = 8.25 mM⁻¹ cm⁻¹) using a 100 Cary UV-visible spectrophotometer with a Peltier-based temperature controller from Agilent Technologies (Santa Clara, CA). One unit of activity is defined as 1 µmol of substrate reduced per minute. Where indicated, DMTS was replaced with dimethyl disulfide, dibenzyl trisulfide, polysulfide, or sodium tetrasulfide. Polysulfide was prepared as previously described (26). Fd-dependent DMTS reduction activity was measured in the same manner except that Fd replaced methyl viologen and was reduced using titanium citrate. The oxidation of Fd was measured at 425 nm (ε = 13 mM⁻¹ cm⁻¹). *P. furiosus* Fd was purified as described previously (47). The oxygen sensitivity and thermostability experiments were carried out using the methyl viologen-linked DMTS reduction assay. H₂S production was measured using the double-vial system with the methylene blue assay as previously described (16).

**Other methods**

The identification of MBX subunits by MALDI–TOF and LC–MS/MS were performed by the PAMS Facility at the University of Georgia. The production of methanethiol (CH₃SH) and methyl hydrogen disulfide (CH₃S-SH) from DMTS reduction assay were identified as their 5,5'-dithiobis-(2-nitrobenzoic acid) derivatives. The standard DMTS reduction assay was carried out as described above, but the reaction was extended for 30 min at 80 °C before taking the samples from the headspace to interact with 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The reaction mixture was incubated for 1 h at room temperature. The resulting solution was ran on a Bruker Daltonics Esquire 3000 Plus ion trap mass spectrometer using an electrospray source. Metal analysis was carried out using an octopole-based ICP–MS (7500ce; Agilent Technologies) as described previously (21).

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