Structure of the respiratory MBS complex reveals iron-sulfur cluster catalyzed sulfane sulfur reduction in ancient life

Hongjun Yu\textsuperscript{1,3,4}, Dominik K. Haja\textsuperscript{2,4}, Gerrit J. Schut\textsuperscript{2}, Chang-Hao Wu\textsuperscript{2}, Xing Meng\textsuperscript{1}, Gongpu Zhao\textsuperscript{1}, Huilin Li\textsuperscript{1} \textsuperscript{✉} & Michael W. W. Adams\textsuperscript{2} \textsuperscript{✉}

Modern day aerobic respiration in mitochondria involving complex I converts redox energy into chemical energy and likely evolved from a simple anaerobic system now represented by hydrogen gas-evolving hydrogenase (MBH) where protons are the terminal electron acceptor. Here we present the cryo-EM structure of an early ancestor in the evolution of complex I, the elemental sulfur (S\textsubscript{0})-reducing reductase MBS. Three highly conserved protein loops linking cytoplasmic and membrane domains enable scalable energy conversion in all three complexes. MBS contains two proton pumps compared to one in MBH and likely conserves twice the energy. The structure also reveals evolutionary adaptations of MBH that enabled S\textsubscript{0} reduction by MBS catalyzed by a site-differentiated iron-sulfur cluster without participation of protons or amino acid residues. This is the simplest mechanism proposed for reduction of inorganic or organic disulfides. It is of fundamental significance in the iron and sulfur-rich volcanic environments of early earth and possibly the origin of life. MBS provides a new perspective on the evolution of modern-day respiratory complexes and of catalysis by biological iron-sulfur clusters.
Respiratory complexes couple the energy released from spontaneous electron transfer reactions to the generation of chemical gradients. From archaea and bacteria to mammalian mitochondria, modern-day respiratory complex I contains a 14-subunit core that oxidizes NADH, reduces quinone, and translocates four protons across the membrane, used to drive ATP synthesis thereby conserving energy. Such complexes first appeared billions of years ago in an anaerobic environment where, without oxygen, oxidative microbial metabolism ultimately reduced the ubiquitous proton to produce hydrogen gas catalyzed by a membrane-bound [NiFe]-hydrogenase (Fig. 1a). As the atmosphere became more oxidized, ancestral MBH evolved to reduce elemental sulfur ($S^0$), now represented by sulfane sulfur reductase or MBS, and ultimately to reducing quinone, as in complex I and related NDH.

MBH and MBS are found in the anaerobic hyperthermophilic archaeon Pyrococcus furiosus, which oxidizes the redox protein ferredoxin, produces $H_2S$ or $H_2$, and conserves energy by pumping $Na^+$ (Fig. 1a). In the presence of $S^0$, the genes encoding MBS are expressed while those encoding MBH are repressed, resulting in a microbial cell yield twice that when cells are grown without $S^0$. This suggests that MBS is more efficient in energy conservation although the underlying mechanism is unknown. MBS reductively cleaves linear polysulfide chains ($S_n^{2−}$, where $n ≥ 4$), produced abiotically from the reaction of $H_2S$ and $S^0$ (Eq. 1) in the hydrothermal vent environments that P. furiosus inhabits, into two shorter polysulfides (Eq. 2). Further reduction yields unstable di- and trisulfides (Eq. 3) that spontaneously convert to $S^0$ and $H_2S$ (Eq. 4). S–S bond cleavage by MBS does not involve protons (Eqs. 2 and 3).

![Fig. 1 Overall structure of the P. furiosus MBS complex.](https://example.com/fig1.png)

**Fig. 1 Overall structure of the P. furiosus MBS complex.** a Schematic comparison of P. furiosus MBH and MBS complexes, both generating a $Na^+$ gradient for ATP synthesis. b Side (left) and top (right) views of the cryo-EM map of the dimeric MBS complex, segmented and colored by subunits. c The front (left) and back (right) view of the atomic model of an MBS monomeric complex. Individual subunits are colored as in (b). Inset in the right panel: a chain of three [4Fe-4S] clusters in the peripheral arm, with the center-to-center and edge-to-edge distances (in brackets) between metal sites indicated in Å.
and H₂S is produced abiotically (Eq. 4). Bacterial polysulfide reductase (PSR, not present in *P. furiosus*) uses a molybdopterin cofactor to reductively cleave the terminal sulfur of polysulfohydroxamides as H₂S (Eq. 5)²³,²⁴ so the archaeal MBS must reduce a disulfide bond by a different mechanism.

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\text{Abiotic : } S_{8} + H_{2}S \rightarrow S - S - S - S - S - S - S - S - S - S - S (S_{2}^{2-}) + 2 H^{+} \\
\text{MBS : } S_{2}^{2-} + 2 e^{-} \rightarrow S - S - S - S - S^{+} + S - S - S - S - S - S (S_{2}^{2-}) \\
\text{MBS : } S_{2}^{2-} + 2 e^{-} \rightarrow S - S - S^{+} + S - S - S - S - S - S (S_{2}^{2-}) \quad (3)
\]

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\text{Abiotic : } 5 S_{2}^{2-} + 4 H^{+} \rightarrow S_{5} + 2 H_{2}S \quad (4)
\]

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\text{PSR : } S_{n}^{2-} + 2 e^{-} + 2 H^{+} \rightarrow S_{n-1}^{2-} + H_{2}S \quad (5)
\]

The structure of MBH (298 kDa, 14 subunits) revealed its close evolutionary relationship with complex I. Although MBH contains a single proton pump compared to four in complex I, they have the same structural elements thought to enable transduction of redox energy to an ion gradient.⁶,⁷,²⁵,²⁶ Herein we describe the structure of MBS (357 kDa, 13 subunits), the proposed evolutionary link between them⁹, and how a catalytic site evolved from reducing protons to reducing large hydrophobic molecules like S₈²⁻ and cleaving an inorganic sulfur–sulfur bond, a reaction of high significance in the primordial early earth.

**Results**

**Structure of MBS.** We determined the cryo-EM structure of MBS in a dimer complex form (Fig. 1b, c, Supplementary Fig. 1, Supplementary Fig. 2, Supplementary Table 1). The atomic model contained 26 protein subunits and 116 transmembrane helices (TMHs). The MBS monomer consists of a peripheral cytoplasmic arm (Mbs), K, L, and N), anchored to the membrane by MbsM, and coordinates three [4Fe-4S] clusters that transfer electrons from ferredoxin to the substrate polysulfide (Fig. 1c)¹⁹. The membrane arm (MbsA, B, C, D, E, G, H, H', and M) has 58 TMHs and contains a module proximal to the peripheral arm (proximal membrane module; MbsH'), MbsH, MbsD, MbsG and TMH1-2 of MbsE and a distal membrane module (MbsA, B and C, and TMH3-6 of MbsE) (Fig. 2a, Supplementary Fig. 3a).

The MBS dimer complex is assembled via extensive contacts at two main places between the proximal membrane module of one MBS and the membrane-anchored peripheral module of the other (Figs. 1b, 2a, b–c). The first contact involves a long amphipathic horizontal helix (termed helix HL) of MbsH' (Fig. 2d): the MbsH' helix HL of one MBS interacts extensively with the N-terminal region of MbsN and TMH8 of MbsM in the other MBS, including several hydrophobic interactions and a salt bridge. The second contact involves the tryptophan-rich C-terminal extension (W90, W91, and W94) of MbsD of one MBS projecting into a largely hydrophobic cleft in the peripheral domain of the other (Fig. 2e). The distal membrane module is not involved in the MBS dimer interface and is likely somewhat dynamic, which may explain the lower local resolution in this region (Supplementary Fig. 1g).

**MbsH and MbsH' each contain a proton pump.** In the proximal membrane module, the two largest subunits MbsH and MbsH' are of similar fold, consistent with the idea from sequence comparison that MbsH' is a gene-duplication product of MbsH¹⁹ (Supplementary Fig. 3b) that occurred prior to the evolution of MBH and MBS and, as discussed below, during the evolution of the Na⁺/H⁺ Mrp antiporter. MbsH and MbsH' are homologous to the antiporter-like subunits of complex I (*T. thermophilus* Nqo12, 13 and 14) proposed for H⁺ pumping—³,³⁴ MbsH and MbsH' each contain two five-TMH repeating units: TMHs 4-8 and TMHs 9-13 that are superimposable when one repeat is flipped upside down in the membrane (Supplementary Fig. 3b). Each 5-TMH unit features a discontinuous α-helix—TMH7 in the first repeat and TMH12 in the second repeat with conserved charged residues around the helical kink: E219 and K393 in MbsH, and K217 and K399 in MbsH'. Notably, the arrangement between MbsH and MbsH' as a unit is strikingly similar to Nqo13 and Nqo14 of *T. thermophilus* complex I (Fig. 3a, b). As a result, a central charged axis across MbsH and MbsH' (MbsH E138, K250, and H330; MbsH' E128, K248, and H336) is formed that is conserved in complex I (Fig. 3c). These features are characteristic of the proton pumps that are found in mammalian and bacterial forms of complex I, which have been supported by structural studies, mutagenesis analysis, and recent molecular dynamics simulations—³,⁻²⁸,⁻³⁰. We therefore propose that MbsH and MbsH' each contains a H⁺ pump in MBS.

Interestingly, MbsH' contains two extra TMHs (TMH115 and TMH116) that are connected by a long intervening loop (Loop₅₇,₂₅₋₆) and the horizontal helix HL. The helix HL and its following TMH16 holds MbsH and MbsH' together. This element is conserved across the different respiratory complexes, with the helix HL either shortened in MBH (with one antiporter-like subunit MbhH) or elongated in complex I (with three antiporter-like subunits) (Fig. 3a, b, Supplementary Fig. 4a), strongly supporting the modular evolution of these respiratory machineries.¹¹ Notably, Loop₅₇,₂₅₋₆ and TMH15 of the C-terminal end of MbsH' tie together the membrane-anchored peripheral module and the proton-pumping module (Supplementary Fig. 4a). This organization mode is conserved in the MBH complex, though MbsM and MbsH' form a tighter interacting interface than the lipid-filled wide cleft between MbhM and MbhH in the MBH complex (Supplementary Fig. 4b)⁶. In contrast, in complex I, the membrane-anchored peripheral module is located on the opposite side of the membrane arm (Fig. 3a). However, in spite of this different architecture, a charged axis within the membrane-anchored peripheral arm (MbsM E203, E211, D196, E193, and E104) extends continuously into the proton-pumping module (MbsH and MbsH') (Fig. 3c), similar to that proposed in complex I for energy transduction—³,³².

The tentative Na⁺/H⁺ antiporter module. Except for MbsH and MbsH', the remainder of the proximal membrane module of MBS is composed of two layers of helix bundles next to MbsH: one layer composed of three TMHs of MbsD and the other layer composed of five TMHs—TMH1-2 of MbsE separated by TMH1-3 of MbsG in the middle (Supplementary Fig. 3a). This subregion together with MbsH is conserved among MBH and complex I including key elements: charged residues E58 in MbsD, E37 in MbsG, and a π-bulge distortion in TMH3 of MbsD (Supplementary Fig. 4a and c). These are key features proposed for proton transfer that are conserved in Ngo10 and 11 in complex I and also in Mrp, Mbh, and G and E in MBH2,²,⁶,³³, although the precise mechanism is still under debate.¹,²,³³ This suggests a putative proton translocation path, termed Pc path for composite proton translocator. We note that the assignment of the Pc proton path is based on structural conservation among MBH, MBS, and complex I, and therefore, this path is tentative. The translocation stoichiometry of the H⁺ and Na⁺ is currently unknown and has yet to be experimentally determined with either Mrp, MBH, or MBS. The stoichiometry will help to determine if Pc is a functional proton path.
In the distal membrane module of MBS, MbsA at the far end contains two TMHs and a following ferredoxin-like domain and binds to the three 3-TMH subunits MbsB, C, and TMH3-6 of MbsE, which in turn are attached to MbsD of the proximal membrane module (Supplementary Fig. 3a). Notably, MbsB, C, and E contain three conserved regions that are rich in charged/polar residues and run across the interior of the membrane arm (Supplementary Fig. 4e). In addition, the conserved MbsC P83 introduces a break in the middle of the membrane of MbsC TMH3. These features are conserved in the Na\(^{+}\)-translocation path in MBH and in Mrp, but not in complex I as complex I does not translocate Na\(^{+}\) ions (Fig. 3a, Supplementary Fig. 4d)\(^{6,34}\). This suggests a Na\(^{+}\)-translocation path within this sub-region. This together with the adjacent H\(^{+}\) path (Pc) likely forms the Na\(^{+}\)/H\(^{+}\) antiporter module in the distal end of the membrane arm (Fig. 3c).

During the review of this manuscript, the structure of Mrp antiporter was published\(^{35}\). The structure enables a comparison between MBS and Mrp to obtain direct evolutionary insights between them (Fig. 4a). All subunits of the Mrp Na\(^{+}\)/H\(^{+}\)
antiporter have homologous counterparts in MBS (Supplementary Table 2). Consequently, the MBS membrane arm (excluding MbsM, which anchors the peripheral arm) shares a conserved architecture with Mrp (Fig. 4a). It is clear that the Mrp structure lacks the membrane-anchored peripheral arm and supports the modular evolution of respiratory complexes31. The emergence of MBS and MBH was likely a result of the combination of the Mrp antiporter and a peripheral module related to a cytoplasmic [NiFe] hydrogenase. MBH exhibits loss of the MrpA homolog resulting in fewer proton pumps while its peripheral arm retains the proton reduction activity. In contrast, MBS keeps all the Mrp homologs while its peripheral arm shows concomitant changes in the peripheral module to enable catalysis of the reduction of polysulfide rather than protons (Fig. 4b). Conceivably, both MBS and MBH are much more efficient in energy conservation as the redox reaction is now physically coupled with the ion translocation across the membrane.

A three-loop system bridges the electron transfer site and the ion-pumping membrane arm. The architecture of the MBS cytoplasmic module closely resembles those of MBH and complex I (Supplementary Fig. 5). In MBS, a large hydrophobic chamber, similar in size to those in MBH and complex I, is formed by cytoplasmic MbsL, MbsJ, and their membrane anchor MbsM (Figs. 5a–c, 6a). The openings of the MBS and complex I hydrophobic chamber are wider than that of MBH allowing entry of hydrophobic substrates (polysulfide and quinone; Fig. 5d). The end of this chamber in MBS coincides with the binding site of the quinone headgroup defined by Nqo4 H38 and Y87 in complex I.
However, these two residues for quinone headgroup binding and also for protonation in complex I are absent in MBS (Fig. 5f). Moreover, MbhL E21, the key residue for proton reduction in the MBH complex, is also absent in MBS6. These suggest a unique catalytic mechanism for MBS despite similar modular overall architecture, as we will cover in the next section.

This hydrophobic chamber containing the catalytic site of MBS in the peripheral arm is defined by three loops originating from MbsH’ (loopTMH15-16, now termed Loop 1), MbsL (loopβ1–β2, Loop 2), and MbsM (loopTMH5-6, Loop 3) (Fig. 6b). The three-loop cluster connects the electron-transferring peripheral arm to the ion-pumping membrane arm and is strikingly similar in the three complexes (Fig. 6b)1–3,6. In MBS, Loop 1 via TM15 and TM16 of MbsH’ connects to its horizontal helix HL that spans two proton pumps (MbsH and MbsH’) (Supplementary Fig. 6a). Similarly in MBH, Loop 1 connects TM1 and TM2 of MbhL to its shorter helix HL that spans a single proton pump (MbhH). In complex I, Loop 1 and its preceding TM1 of Nqo7 locate the conserved membrane-anchored peripheral module to a different site of the membrane arm via TM2-3 of Nqo7 while the much longer helix HL of Nqo15 that spans three proton pumps (Nqo12, 13, and 14) is similar to MBS and MBH (Supplementary Fig. 4a).

A central charged axis in MBS extending from the three loops across the membrane arm in parallel to helix HL is also found in both complex I and MBH (Figs. 3c, 6c)1–3,6,32. Clearly, the structural elements in MBH and complex I necessary for energy conservation are maintained in MBS. Therefore, we suggest that Loop1 and its preceding TM are a key structural feature that has been conserved across evolution in order to sustain the fundamental function of coupling the redox reaction in the peripheral arm with ion translocation in the membrane arm.

The peripheral arm of MBS contains a uniquely coordinated [4Fe-4S] cluster. In MBS, the three [4Fe-4S] clusters form an efficient electron transfer pathway to the chamber from Fd (Fig. 1c inset)36. MbsN coordinates the distal and the medial [4Fe-4S] clusters (relative to the catalytic chamber; Supplementary Fig. 6a). The proximal (p-) [4Fe-4S] cluster in MBS and complex I is at the top of the hydrophobic chamber (Supplementary Fig. 6b) but, in stark contrast to that in complex I (and MBH), the MBS p-[4Fe-4S] is coordinated by only three rather than the expected four cysteines (C28, C93 and C123 of MbsJ; Fig. 6d).

(Fig. 5e)1. However, these two residues for quinone headgroup binding and also for protonation in complex I are absent in MBS (Fig. 5f). Moreover, MbhL E21, the key residue for proton reduction in the MBH complex, is also absent in MBS.8 These suggest a unique catalytic mechanism for MBS despite similar modular overall architecture, as we will cover in the next section.
The different functions of the p-[4Fe-4S] clusters in these enzymes (Fig. 5b) are determined by a conserved structural motif containing a short N-terminal α-helix, a β-strand, a variable loop with two cysteines and another short α-helix (in MbsJ for MBS complex) (Supplementary Fig. 6b, c). In MBH and cytosolic [NiFe] hydrogenases6,37, the two cysteines are two residues apart and ideally positioned to both coordinate the proximal cluster, which donates electrons to the NiFe-catalytic site. In complex I, the two cysteines (C45 and C46 in Nqo6) are adjacent to each other enabling sequential dissociation upon proximal cluster reduction, which has been proposed to facilitate electron and proton transfer to the quinone although this is under debate16,38,39. In MBS, this structural motif is three residues shorter than it is in MBH, leaving one of the cysteines (MbsJ C25) no longer able to participate in cluster coordination (Fig. 6d, Supplementary Fig. 6c). Hence, one iron atom of the p-4Fe-4S] cluster in MBS has non-cysteinyl coordination (Supplementary Fig. 2j) and therefore catalytic potential. Besides the three [4Fe-4S] clusters, MBH has an additional proton-reducing [NiFe]-site, which is coordinated by four cysteines of MbhL (Supplementary Fig. 6d)6,40. Of these four cysteines, two are not present in MBS, leaving the two conserved residues (C85 and C385 in MbsL) adjacent to the proximal cluster (Fig. 7a). Complex I has lost all four, supporting the stepwise evolution of MBS and complex I by

Fig. 5 A comparison of the chamber at the active sites of MBS, MBH and complex I. The structures were overlaid based on MbsM, Nqo8, and MbhM and are shown individually. The chamber is similarly formed at the interface between the peripheral arm and its membrane anchor. The black arrow indicates the opening of the chamber proposed as the entrance of quinone in complex I studies. a MBS. b Complex I (PDB ID 4HEA). c MBH (PDB ID 6CFW). A comparison of the chamber entry between MBS, MBH, and complex I indicate a wider opening in MBS and complex I than that in MBH. e Zoomed view of the end of the active site chamber, outlined as the boxed region in (a). The aligned Nqo4 of Tt complex I is shown as a green cartoon where two residues (H38 and Y87) proposed for the binding of the quinone headgroup are shown as sticks. f Sequence alignment of MbsL with its counterparts in different respiratory complexes (Nqo4 of Tt complex I, NdhH of NDH complex, MbhL of MBH complex I). Residues equivalent to Nqo4 H38 and Y87, proposed for quinone headgroup binding and protonation in complex I, and MbhL E21, the key residue for proton reduction in MBH complex, are all absent in MBS.
sequential loss of the four cysteines coordinating the [NiFe] center in MBH.

In MBS, two potential polysulfide-reducing sites are close (~12 Å) to the p-[4Fe-4S] cluster: MbsJ C25 and MbsL C85/C385 (Fig. 7a). However, they are not involved in catalysis as the C25A MbsJ and the C85A/C385A MbsL mutant enzymes had comparable catalytic activities using the model substrate dimethyltrisulfide (DMTS) to that of the wild-type (WT) enzyme (1.8 ± 0.4 units/mg) (Fig. 7b). Cluster interconversion was used to provide evidence that the p-[4Fe-4S] cluster of MBS is itself reducing polysulfide, and doing so with a specific role for its unique non-cysteinyl coordinated Fe atom. The WT-enzyme was treated with potassium ferricyanide, a well-established procedure to remove non-cysteinyl coordinated Fe atom from an [4Fe-4S] cluster to generate a [3Fe-4S] cluster. This caused an ~98% loss of activity (Fig. 7b). Subsequently incubating the enzyme under reducing conditions with ferrous iron, conditions that are known to restore the [4Fe-4S] cluster from a [3Fe-4S] cluster, the MBS activity was restored to its original value (Fig. 7b).

Further evidence for the proposed catalytic p-[4Fe-4S] cluster came from EPR spectroscopy. MBS from S0-grown cells gave rise to an intense free-radical EPR signal ($g = 2.00$) unaffected by chemical reduction (Supplementary Fig. 7a) but this was a puriﬁcation artifact because the enzyme from non-S0 grown cells (in which transcription of the genes encoding MBS was controlled by a non-regulated promoter) did not exhibit this EPR signal (Supplementary Fig. 7b). Chemical reduction of MBS did lead to the appearance of a broad EPR signal ($g \approx 1.94$), consistent with three interacting and reduced [4Fe-4S] clusters (Supplementary Fig. 7b). After treatment with excess ferricyanide, the inactive enzyme (Fig. 7b) exhibited a complex EPR signal indicative of an oxidized $S = 1/2$ [3Fe-4S]$^+$ cluster ($g = 2.09$, Supplementary Fig. 7c), supporting the conclusion that the p-[4Fe-4S] cluster is catalytic and that activity is reversibly lost upon removal of its unique iron. Importantly, chemically reduced P. furiosus MBH...
**Figure 1.**

**a.** Structural visualization of the MbsWT complex with FeCN and FeCN + Fe. The blue ribbon represents the MbsWT complex, while the green ribbon indicates the proximal [4Fe-4S] cluster. The black arrows indicate the electron transfer pathways.

**b.** Graph showing specific activity (U/mg) for different conditions: MbsWT, MbsWT + FeCN, MbsL C85A, and MbsL C85A/C385A. The bars indicate standard error measures.

**c.** Reaction pathway for the oxidation of sulfur. The reactions involve the transfer of electrons from sulfur species to the iron-sulfur clusters (m-[4Fe-4S] and p-[4Fe-4S]). The reaction is driven by the production of QH₂ and H₂ via NiFe.

**d.** Summary of the electron transfer reactions involving [4Fe-4S] clusters. The reactions include the oxidation of sulfur species and the production of Q and 2H⁺ via NiFe.
gave rise to an EPR signal (Supplementary Fig. 7d) similar to that of reduced MBS (Supplementary Fig. 7b), but when treated with ferricyanide under the same conditions used for MBS, no activity was lost and the MBH sample was EPR silent, consistent with the presence of only oxidized [4Fe-4S] clusters (Supplementary Fig. 7e). Unlike MBS, the p-[4Fe-4S] cluster of MBH is only involved in electron transfer and has full cysteinyl coordination, hence it is not susceptible to inactivation due to iron loss and oxidative cluster conversion.

The proximal [4Fe-4S] cluster uses a novel, ancestral mechanism for sulfur reduction. The reduction of polysulfide by the p-[4Fe-4S] cluster in the hydrophobic pocket of MBS (Fig. 7a) is the first example in a biological system of reduction of an inorganic disulfide bond without the release of H2S (Eqs. 2 and 3, c.f. Eq. 5). Three types of enzymes use iron-sulfur clusters to indirectly facilitate disulfide bond cleavage; heterodisulfide reductase, hydrogenase processing protein HydD, and ferredoxin thioredoxin reductase (FTR). Based in part on FTR, we propose a new mechanism for the reduction of an internal disulfide (which could be either inorganic or organic) by MBS (Fig. 7c). The unique Fe atom of its p-[4Fe-4S]2+ cluster binds polysulfide (S2n−), accepts an electron from the medial (m-)[4Fe-4S]1+ cluster (Fig. 1c) and catalyzes the two-electron reduction of an internal disulfide, generating two smaller polysulfides (S0n−2 and S0n−3) and the superoxidized form of p-[4Fe-4S]3+. As in FTR, the reduction of the p-[4Fe-4S]2+ cluster actually leads to its superoxidation because two electrons are used to reduce the disulfide. Moreover, the cluster must be in the 3+ (rather than 2+) state to bind the two product polysulfides at the unique Fe site (Fig. 7c). The catalytic cycle is completed by reduction of p-[4Fe-4S]3+ by a second electron from m-[4Fe-4S]1+ (after its reduction by the distal [4Fe-4S]1+ cluster) and release of the two smaller polysulfides. This is the simplest mechanism yet proposed for the reduction of an S-S bond in either an inorganic or organic sulfide and is the first not requiring either protons or amino acid residues. This reaction is clearly of fundamental significance in iron and sulfur-rich hydrothermal vent environments of the early earth and possibly in the origin of early life, as well as in the subsequent evolution and expansion of iron-sulfur cluster functionality in the diverse array of modern-day iron-sulfur proteins.

That catalysis by MBS does not require water or protons (Fig. 7c) is consistent with a highly hydrophobic pocket and the absence in MbsL of the well-defined proton transfer pathway to the NiFe site found in MbHL and other hydrogenases. The requirement for binding two polysulfides (Fig. 7b) is super-oxidation of the p-[4Fe-4S] cluster (3+), which is facilitated by the hydrophobic site, analogous to superoxidation of hydrophobic [4Fe-4S] clusters in high potential iron-sulfur proteins (HiPIPs). The spontaneous breakdown of tri- and disulfides to release H2S does require protons (Eq. 4) and must occur in the cytoplasm rather than the hydrophobic catalytic site (Fig. 7c). The di- and trisulfides produced by MBS do not generate H2S (or HS−), are stable in the hydrophobic pocket (where there are no protons or water), and H2S is only generated when they are released from the enzyme (Fig. 7c).

Discussion

The standard state ΔG (pH 7.0) of the electron transfer reactions for MBS (41 kJ/mol/2e−) is more than twice that available to MBH (12 kJ/mol/2e−), and half of that available to complex 1 (81 kJ/mol/2e−)39, consistent with the pumping of two, one and four protons, respectively1,2,7,8,47. The MBH structure confirms that the transduction of redox energy into the spatially separated translocation of ions involves the three-loop cluster1–3,31,48 and a central charged axis1–3,7,32 (Fig. 6b, c), all highly conserved across evolution. The proposed MBS mechanism also shows that there are key fundamental differences in spite of a common overall means of energy transduction. In MBS, oxidation of the reduced m-[4Fe-4S]1+ cluster leads to superoxidation of the oxidized p-[4Fe-4S]2+ cluster (2+/3+ redox couple) but in MBH and complex I to reduction of the oxidized p-[4Fe-4S]1+ cluster (2+/1+ redox couple; Fig. 7c). Hence the loss of the NiFe site of an ancestral MBH through evolution generated a highly hydrophobic cavity enabling both MBS and complex I to use the p-[4Fe-4S] cluster to directly interact with and reduce hydrophobic substrates, albeit using different charge states of the cluster (Fig. 7d).

It is not clear how a change in the redox status of the m-[4Fe-4S] cluster is ultimately coupled to a change in the dynamics of the three-loop cluster, nor how the different amounts of free energy released (12–81 kJ/mol/2e−) lead to corresponding differences in the proton motive force that is generated (1–4 protons). It is interesting to speculate that MBS might conserve more (or less energy) if one proton pump (MbsH+ equivalent, see Fig. 3c) were added (or inactivated, by mutagenesis) and that the cell yield of P. furiosus growing on S0 would be correspondingly higher (or lower). Such experiments are in progress. On a more fundamental level, the question yet to be answered is, as illustrated in Fig. 7d, how does m-[4Fe-4S]1+ oxidation coupled to a change in the conformation of the three-loop cluster lead to the conservation of different quantities of energy in these three types of the respiratory complex? In any event, the simple aprotic and abiotic catalytic mechanism of S–S bond reduction by MBS might be a modern remnant of an iron–sulfur world41 as well as the precursor to more complex mechanisms involving iron–sulfur clusters and those now found in the aerobic world, such as the reduction of quinones by complex I.

Methods

Purification of MBS. The MBS holoenzyme (S-MBS) was solubilized and purified anaerobically from Pyrococcus furiosus strain MW0491, in which a His-tag has been engineered at the N terminus of the Mbh subunit19. Frozen cells were lysed in 25 mM sodium phosphate, pH 7.5, containing 1 mM DTT and 50 μg/mL DNase I (5 μL per gram of frozen cells). After stirring for one hour, the cell-free extract was centrifuged at 100,000 × g for 1 h. The supernatant was removed and the membranes were washed twice using 50 mM EPPS buffer, pH 8.0, containing 5 mM MgCl2, 50 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, and 0.1 mM PMSF. The membrane pellet was collected by ultracentrifugation at 100,000 × g for one hour after each wash step. The washed membranes were resuspended in 50 mM Tris-HCl, pH 8.0, containing 5 mM MgCl2, 50 mM NaCl, 5% (v/v) glycerol, 1 mM DTT, and 0.1 mM PMSF. MBS was solubilized by adding n-dodecyl-β-D-maltoside (DDM, Novabiochem) to 3% (w/v) followed by incubation at 4 °C for 16 hours. The solubilized membranes were centrifuged at 100,000 × g for 1 h. The supernatant was applied to a 5 mL His-Trap crude FF Ni-NTA column (GE Healthcare) while diluting it 10-fold with buffer A (25 mM sodium phosphate, 300 mM NaCl, pH 7.5, containing 1 mM DTT and 0.03% DDM). The column was washed with 10 column volumes of buffer A and the bound protein was eluted with a 20-column volume gradient from 0 to 100% buffer B (buffer A containing 500 mM imidazole). The eluted protein was further purified by applying it to a 1-mL His-Trap FF Ni-NTA column (GE Healthcare) while diluting it 5-fold with buffer A. A 30-column volume gradient from 0 to 100% buffer B was used to elute the bound protein. The MBS sample was concentrated and further purified using a Superose 6 10/300 GL column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.2, containing 300 mM NaCl, 2 mM sodium dithionite, and 0.03% DDM.
**P. furiosus strain construction.** The strains used in this study are summarized in Supplementary Table 3. The genetically tractable *P. furiosus* strain COM1 was used for the expression and the assay procedures of MBS8. After confirming its growth in a half-life of 19 h using overlapping PCR. The upstream flaunting region (UFR), which also contained the selection marker (Pbaa-prfP) and the promoter of the gene encoding the S-layer protein (Pab), and the downstream flaunting region (DFR), were amplified from MW0567 gDNA. Mutagenesis was carried out using site-directed mutagenesis, with primers designed to amplify the MbsC25A mutation. The primers used are listed in Supplementary Table 4. The fragments were assembled using overlapping PCR and the insertion cassette was transformed into the intergenic space between PF0265 and PF0266 of MW0011. The transformants were grown as previously described and the PCR-confirmed colonies were sequence verified using Sanger sequencing (Geneviz).

**Puriﬁcation of C-MBS, activity assays, and EPR methods.** The three C-MBS subcomplexes (WT, MbsC25AS/C38S, MbsC25A) were puriﬁed anaerobically as described previously. Frozen cells were lysed in 25 mM sodium phosphate, pH 7.5, containing 1 mM DTT and 50 μg/μL DNase I (5 μL per gram of frozen cells). After stirring for one hour, the cell-free extract was centrifuged at 100,000 x g for 1 hour. The supernatant was applied directly to a 5 mL His-Trap FF-Ni-NTA column (GE Healthcare) by diluting it 10-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 5 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). All samples were carried out at 80 °C using anaerobic sealed vials as described previously. The dimethyl trisulfide (DMTS) reduction assay used a 2 mL reaction mixture containing 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.5, and 150 mM NaCl. After pre-heating to 80 °C, 1 mM methyl viologen reduced by titanium citrate and 2 mM DMTS were added and the reaction was induced by the addition of the enzyme. For inhibition by enzyme inversion, the enzyme (2 mg/mL in 25 mM sodium phosphate, pH 7.5) was incubated anaerobically with potassium ferricyanide (1 mM) for ~90 h at room temperature. The sample was then buffer exchanged to remove excess ferricyanide before measuring activity. To activate the enzyme, a mixture of sodium dithionate (1 mM) and Fe(II) (1 mM) were added and after ~90 h at room temperature, the sample was buffer exchanged to remove the excess reagent. Enzyme activity was measured by the reduction of DMTS by monitoring the oxidation of methyl viologen at 600 nm (ε = 8.25 mM⁻¹ cm⁻¹) as previously described. One unit of activity is defined as 1 μmol of DMTS reduced per minute.

**Cryo-EM analysis and data acquisition.** MBS was puriﬁed using detergent DDM and was concentrated to ~5 mg/mL. Cryo-EM grids were prepared under the aerobic conditions in air without DDM (used in a half-life of 19 h). In brief, three-microliter aliquots of the sample were applied to glow-discharged Quantifoil R 1.2/1.3 gold grids (300 mesh). The grids were blotted for 3 s at 10 °C with 100 kV FEI Toloform Mark IV device. Cryo-EM data were recorded on a K2 camera positioned post a GIF quantum energy filter in a 300 kV FEI Titan Krios electron microscopy. Two datasets were automatically collected with Serial EM 3.7beta and FEI EPU software package, spanning a map with an overall resolution of 4.0 Å when twofold symmetry was applied. We note that most regions, particularly in the peripheral arm and the proximal membrane module, are of substantially better resolution in the range of 3.0-3.5 Å. Some strategies including applying C1 symmetry, masking out detergent micelle or excluding the symmetry failed to improve the map. We therefore reasoned that the low overall resolution (4 Å) may be due to the partially flexible distal membrane region. The resolution of the reconstructed map was estimated based on the gold-standard Fourier shell correlation 0.143 criterion. The final map was corrected for the modulation transfer function of the detector and sharpened by applying a negative B-factor, estimated by the post-processing procedure in RELION-3.0. The estimate of Local resolution distribution was calculated using ResMap 1.1.57.

**Model building and reﬁnement.** The initial models of the distal module (MbsA-C, MbsE TMH3-6) and membrane-anchored hydrogenase module (MbsJ-N) were generated with the SWISS-MODEL server using the structure of *P. furiosus* MBH complex (PDB ID 6CFW) as the template. The initial model of the proximal module (MbsD, MbsE TMH3-6, MbsG, MbsH, and MbsH) were generated using the structure of *T. thermophilus* complex 1 as a template (PDB ID 4HEA). These models were individually rigid-body docked into the 3D density map using the Fit-in-map function in Chimera 1.8. These fitted models were improved by manual adjustment and rebuilding in Coot 0.8.99. The rebuilding process was aided by the good density features of a helices and many bulky residues such as Phe, Trp, Tyr, and Arg. The clusters within the peripheral arm were modeled based on their electron densities and also the homology of cluster-coordinating subunits with complex I and MBH. The real-space reﬁnement of the MBS complex model against the cryo-EM map was performed using the phenix.real_space_refine in PHENIX 1.15.61. MolProbity 4.1 (Duke University) was used to assess the final model.92 Chimera and PyMOL 1.8 (Schrödinger, LLC) were used to prepare the Figures. Statistics of the 3D reconstruction and model reﬁnement were provided in Supplementary Table 1. Since all the subunits of Mrp antiporers have their counterparts in MBS (Supplementary Table 2), the structure of *B. subtilis* Mrp antiporers was modeled based on the MBS structure using the SWISS-MODEL server55.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The accession number for the atomic coordinates reported in this paper is PDB ID 6USY. The accession number for the EM density map reported in this paper is EMD-20692. Source data are provided with this paper. Other data are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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