Protein complexing in a methanogen suggests electron bifurcation and electron delivery from formate to heterodisulfide reductase

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In methanogenic Archaea, the final step of methanogenesis generates methane and a heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB). Reduction of this heterodisulfide by heterodisulfide reductase to regenerate HS-CoM and HS-CoB is an exergonic process. Thauer et al. [Thauer, et al. 2008 Nat Rev Microbiol 6:579–591] recently suggested that in hydrogenotrophic methanogens the energy of heterodisulfide reduction powers the most endergonic reaction in the pathway, catalyzed by the formylmethanofuran dehydrogenase, via flavin-based electron bifurcation. Here we present evidence that these two steps in methanogenesis are physically linked. We identify a protein complex from the hydrogenotrophic methanogen, Methanococcus maripaludis, that contains heterodisulfide reductase, formylmethanofuran dehydrogenase, F420-nonreducing hydrogenase, and formate dehydrogenase. In addition to establishing a physical basis for the electron-bifurcation model of energy conservation, the composition of the complex also suggests that either H2 or formate (two alternative electron donors for methanogenesis) can donate electrons to the heterodisulfide-H2 via F420-nonreducing hydrogenase or formate via formate dehydrogenase. Electron flow from formate to the heterodisulfide rather than the use of H2 as an intermediate represents a previously unknown path of electron flow in methanogenesis. We further tested whether this path occurs by constructing a mutant lacking F420-nonreducing hydrogenase. The mutant displayed growth equal to wild-type with formate but markedly slower growth with hydrogen. The results support the model of electron bifurcation and suggest that formate, like H2, is closely integrated into the methanogenic pathway.

energy conservation | Archaea | formate dehydrogenase | formylmethanofuran dehydrogenase | F420-nonreducing hydrogenase

The biochemical steps in methanogenesis from CO2 are well known, but the interactions that lead to net energy conservation are not well understood. The steps in the pathway are diagrammed in Fig. 1 (1). The first step involves the reduction of CO2 and covalent attachment to a unique cofactor, methanofuran (MFR), via the action of formylmethanofuran dehydrogenase (Fwd) to generate formyl-MFR. This represents an energy-consuming step in the pathway and is dependent on reduced ferredoxin, thought to be produced at the expense of a chemiosmotic membrane potential via the energy-conserving hydrogenase, Eha. Next, the formyl group is transferred to yet another carrier, tetrahymethanopterin (H4MPT), and is then reduced to generate methyl-H4MPT. The methyl group is then transferred to yet another carrier, coenzyme M (HS-CoM), by methyl-H4MPT-CoM methyltransferase (Mtr) to generate methyl-S-CoM. At this point, Na+ ions are translocated across the cell membrane. The final step involves reduction of the methyl group to CH4 and capture of HS-CoM by coenzyme B (HS-CoB) to form a CoM-S-S-CoB heterodisulfide. To regenerate HS-CoM and HS-CoB, another enzyme is used, heterodisulfide reductase (Hdr).

Most methanogens can use H2 as the electron donor, and many can also use formate. Reduced coenzyme F420 (F420H2) is a required intermediate, and can be generated from H2 by F420-reducing hydrogenase (Fru) or by a cycle involving the enzymes H2-dependent methane-H4MPT dehydrogenase and F420-dependent methylene-H4MPT dehydrogenase (2). When formate is the electron donor, it is oxidized to CO2 by a formate dehydrogenase (FdH) that yields F420H2.

How net energy is conserved in most methanogens is not well understood, because the membrane potential generated during the methyl transfer from H4MPT to HS-CoM would appear to be depleted by Eha to fuel the reduction of CO2 to formyl-MFR. The solution to this dilemma apparently resides in the exergonic heterodisulfide reduction step. Methanogens from the order Methanosarcinales, known as the methylotrophic methanogens, have a membrane-bound electron transport chain involving the quinone-like methanophenazine and a cytochrome-containingHdr complex that translocates protons across the cell membrane concomitant with CoM-S-S-CoB reduction, resulting in net energy conservation (1). However, all other methanogens (the hydrogenotrophic methanogens) lack methanophenazine and cytochromes, have a cytoplasmic Hdr, and are not known to generate a membrane potential at this step (1). Nevertheless, these organisms grow rapidly and are found in numerous anaerobic environments. It was recently proposed that methanogens without cytochromes use flavin-based electron bifurcation from Hdr to simultaneously reduce CoM-S-S-CoB and reduce ferredoxin for Fwd to generate formyl-MFR (1). If this were to occur, then ferredoxin reduction by Eha would not be required and net energy conservation would result.

It was our intention to find protein–protein interactions involving Hdr that may indicate if there are potential pathways for energy conservation that have eluded prior characterization in methanogens without cytochromes. To this end, we performed experiments with the hydrogenotrophic methanogen, Methanococcus maripaludis. M. maripaludis is ideal for such an undertaking because of its rapid growth under laboratory conditions, a well-developed set of genetic tools (3, 4), the ability to grow in continuous culture under conditions of defined nutrient limitation (5), and the availability of an exhaustive dataset from quantitative measurements of the proteome (6, 7).
+formate His-tagged FdhA1 must be functional. Of containing His-tagged HdrB2 and a null mutation in genome. Growth experiments showed that each His-tagged protein complexes with Hdr. Therefore, we also constructed protein interactions of each. In addition, we had preliminary evidence that reduced formate or hydrogen to Hdr may drive the reduction of the CoM-S-S-CoB heterodisulfide, as well as the reduction of ferredoxin via flavin-mediated electron bifurcation as outlined by Thauer et al. (1). Eha, energy-conserving hydrogenase; Fdh, formate dehydrogenase; Fru, F420-reducing hydrogenase; Fwd, formyl-MFR dehydrogenase; Hdr, heterodisulfide reductase; Hmd, H2-dependent methylene-H4MPT dehydrogenase; Mcr, methyl-CoM reductase; Mer, methylene-H4MPT reductase; Mtd, F420-dependent methylene-H4MPT dehydrogenase; Mtr, methyl-H4MPT-CoM methyltransferase; Vhu, F420-nonreducing hydrogenase.

**Results**

**Hdr Complexes with F420-Nonreducing Hydrogenase, Fdh, and Fwd.** To characterize the protein interactions that take place between Hdr and associated proteins, the β subunits of Hdr were C-terminally tagged with a 10-amino acid extension containing a 6×-His tag. The β subunits were used for purification as this subunit has been demonstrated to contain the active site for heterodisulfide reduction (8). The *M. maripaludis* genome encodes two Hdrs (9), and either HdrB1 (strain MM1263) or HdrB2 (strain MM1264) was tagged to determine if there were differences between the protein interactions of each. In addition, we had preliminary evidence (based on early purification experiments) that one of two FdhIs encoded in the *M. maripaludis* genome might be included in protein complexes with Hdr. Therefore, we also constructed a strain (MM1265) in which FdhA1 was C-terminally tagged with a 13-amino acid extension containing a 6×-His tag. To avoid any confounding influence of the second Fdh, Fdh2, the FdhA1 His-tag was constructed in a background strain that contained an in-frame deletion of the fdh2 gene cluster. In each case, the His-tagged version of the protein replaced the wild-type gene in the genome. Growth experiments showed that each His-tagged protein was functional (Fig. S1). Thus, a strain containing His-tagged HdrB1 and a null mutation in hдрB2 grew normally, as did a strain containing His-tagged HdrB2 and a null mutation in hдрB1. Because Hdr is essential, each His-tagged protein must be functional. Similarly, MM1265 containing His-tagged FdhA1 and a deletion of fdh2 grew normally on formate. Because Fdh is required for growth on formate, His-tagged FdhA1 must be functional.

For protein preparations, three experimental strains were grown: MM1263 containing His-tagged HdrB1, MM1264 containing His-tagged HdrB2, and MM1265 containing His-tagged FdhA1. Two control strains were also grown: the parental strain MM901 containing no His-tagged proteins, and strain MM1262 containing the deleted fdh2 and no His-tagged proteins. All five strains were grown under three conditions: hydrogen excess or limitation in a chemostat and batch culture with formate as the sole electron donor. This process was followed because several genes are regulated in response to hydrogen availability in *M. maripaludis* (10), and there could be differences in the composition of the Hdr complex in response to different growth conditions. Cell extracts were made from all 15 cultures and protein purifications were done anaerobically using Ni-affinity columns. Purified samples were analyzed by mass spectrometry. Spectral counts (SC) were tabulated for any protein that returned ≥10 SC in any of the three growth conditions with the three experimental strains (Table S1).

For each protein, SCs were compared. From an initial inspection of the data it appeared that there were two groups: those that consistently had similar SCs in all five strains (background proteins), and those that had markedly greater SCs in MM1263 and MM1264 (the experimental strains) compared with MM901 (the control strain) or in MM1262 (experimental) to MM1262 (control). To distinguish clearly between these groups, three proteins among the background proteins (reference proteins) were used as the basis for the calculation of normalized SC ratios (Methods). The results are presented in Table S2. A protein was considered enriched by copurification with the His-tagged protein if the normalized SC ratio was greater by at least three SDs than the average ratio for the 13 background proteins, or if more than five SCs were detected in the experimental sample and none was seen in the control. The results are summarized in Table 1. Subunits from five different proteins copurified with both of the His-tagged HdrBs and with His-tagged FdhA1; these were Hdr1, Hdr2, Fdh1, the selenocysteine-containing F420-nonreducing hydrogenase (Vhu), and the tungsten-containing formylmethanofuran dehydrogenase (Fwd). The findings supported these conclusions regardless of which of the three reference proteins was used for the normalization calculation. None of these proteins was observed to bind nonspecifically to His-tagged constructs in *M. maripaludis* (11). In general, multiple subunits of each protein were enriched in the experimental samples, although a few subunits were not detected. Generally, the relative abundances of subunits from each protein were in agreement with those found in proteomic studies of whole-cell extracts from *M. maripaludis* (6, 7), and those subunits that were not detected here were detected at low levels in the whole proteome. The five proteins were enriched in samples from all three growth conditions, except for Fdh1 under H2 excess and all proteins under H2 excess with His-tagged FdhA1. The absence of Fdh in these samples is not surprising because fdh expression is markedly down-regulated when cells are grown under H2 excess (10).

Each of the four enzymes represented in the complex is encoded in the genome in two different forms, but only in the case of the HdrB and HdrC subunits were both purified in the complexes. Thus, both HdrB1 and HdrB2 and both HdrC1 and HdrC2 were generally present in the complex; however, both are not required to make a functional Hdr (Fig. S1). Hdr is a tetramer of trimers in the αβγδ configuration in methanogens (12), and evidently the B1 and B2 subunits are interchangeable, as are the C1 and C2 subunits. In the case of the F420-nonreducing hydrogenase and HdrA, the two forms of the enzyme contain selenocysteine (Vhu and HdrA) or cysteine (Vhc and HdrA). Only the selenocysteine forms were detected here, consistent with previous studies of regulation by selenium, predicting that only the former should be expressed in our selenium-containing medium (13). Indeed, in two studies of the whole proteome of *M. maripaludis*
M. maripaludis, the selenocysteine proteins were detected at least 100-fold more frequently than the cysteine proteins (6, 7). Two formylmethanofuran dehydrogenases are represented in the genome, tungsten-containing (Fwd) and molybdenum-containing (Fmd). Only Fwd was detected here, and in the whole proteome the Fwd subunits were detected at least 10-fold more frequently. Of the two Fdhs, Fdh1 found in the complex with Hdr was also detected at least 10-fold more frequently in the proteome than Fdh2. In contrast, the HdrB and C subunits were detected in more similar amounts in the proteome, with HdrB2 and C2 detected at least 10-fold more frequently than HdrB1 and C1. Hence, the enzyme forms detected in the complexes with Hdr and Fdh1 were consistently those that were more frequently detected in the proteome.

**F_{420}-Nonreducing Hydrogenase Is Not Essential for Growth on Formate.**

The presence of Fdh in a complex with Hdr suggested the possibility of direct electron flow between these two enzymes. If this is the case, then F_{420}-nonreducing hydrogenase might not be needed for growth on formate because H₂ generated from formate (via F_{420}H₂) (2) would not be necessary for heterodisulfide reduction. To test this hypothesis, we constructed in-frame deletions that eliminated the genes encoding the putative active subunits for hydrogen oxidation in both copies of the F_{420}-nonreducing hydrogenase, vhuAU and vhcA. M. maripaludis was transformed with these constructs and a ΔvhAU ΔvhCA mutant (MM1272) was successfully obtained after growth on formate. The mutant grew on formate similarly to the wild-type strain, but grew poorly on H₂ (Fig. 2). The slight growth on H₂ might be explained via a poorly understood F_{420}H₂:heterodisulfide oxidoreductase activity that has been demonstrated for the closely related Methanococcus voltae (14). In any case, F_{420}-nonreducing hydrogenase clearly plays the major role in heterodisulfide reduction with H₂ but is unnecessary for heterodisulfide reduction with formate.

### Discussion

We have reported here evidence for a protein complex that comprises four separate enzymes: heterodisulfide reductase, F_{420}-nonreducing hydrogenase, formate dehydrogenase, and formylmethanofuran dehydrogenase. The coelution of these proteins with three separate protein subunits under three different growth conditions strongly suggests that the observed complex exists in vivo and may be involved in energy conservation. The interaction of Hdr with F_{420}-nonreducing hydrogenase was suggested previously in studies of these enzymes in Methanothermobacter marburgensis (15, 16). Indeed, in some methanogens the α subunit of Hdr is fused to the β subunit of the F_{420}-nonreducing hydrogenase (16). However, the presence of Fdh and Fwd in the complex with Hdr is unique. The results provide insight into electron flow during methanogenesis with formate, and supports the electron bifurcation mechanism for energy conservation during methanogenesis. A model for the role of the protein complex is presented in Fig. 3.

**Fdh–Hdr Interaction.** Previously, only the F_{420}-nonreducing hydrogenase was thought to deliver electrons to Hdr. Hdr purified from M. marburgensis was observed to associate with an F_{420}-nonreducing hydrogenase, but not with Fdh (15, 16). However, this organism is not known to grow with formate as the sole electron donor (17). The association of Fdh with Hdr suggests

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**Table 1. Proteins enriched in purified complexes**

<table>
<thead>
<tr>
<th></th>
<th>H₂-excess*</th>
<th>H₂-limited</th>
<th>Formate</th>
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<tbody>
<tr>
<td></td>
<td>MM1263¹</td>
<td>MM1264</td>
<td>MM1265</td>
</tr>
<tr>
<td>HdrA₀</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>HdrB₁</td>
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<tr>
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<td>ND</td>
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<tr>
<td>FdhA₁</td>
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<td>–</td>
<td>ND</td>
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<tr>
<td>FdhB₁</td>
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<tr>
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<tr>
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<tr>
<td>FwdA</td>
<td>+</td>
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<td>–</td>
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<td>FwdC</td>
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<tr>
<td>VhuA</td>
<td>+</td>
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<tr>
<td>VhuD</td>
<td>+</td>
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<tr>
<td>VhuG</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>VhuU</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

+:+, SC values relative to control samples support enrichment in protein complex (unshaded squares in Table S2).

−: SC values relative to control samples do not support enrichment in protein complex (shaded squares in Table S2).

*Growth condition.

¹Experimental strain. MM1263, His-tagged HdrB1; MM1264, His-tagged HdrB2; MM1265, His-tagged FdhA1.

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**Fig. 2.** Growth of ΔvhU ΔvhC strain vs. wild-type strain on formate or H₂. OD₆₆₀, optical density at 660 nm. (●) MM1272 (ΔvhU ΔvhC) grown on formate; (◆) MM901 (wild type) grown on formate; (▲) MM1272 grown on H₂; (▲) MM901 grown on H₂. Data are from three independent cultures and error bars represent one SD around the mean.
a pathway of electron flow to Hdr that does not involve H₂. Electrons may flow from formate to Hdr via Fdh. In support of this hypothesis, we were able to eliminate Vhu and Vhc and retain rapid growth on formate, although growth was markedly decreased on H₂ (Fig. 2). This finding contrasts with an unsuccessful attempt to delete vhuU in M. voltae (18), even under conditions of sulfur starvation where this organism would theoretically generate a truncated VhuU peptide. However, in that study only H₂, not formate, was used as the electron donor. To our knowledge, thus far our ΔvhuAUΔvhcA mutant is unique among hydrogenotrophic methanogens in having a growth defect specifically on H₂. Its phenotype supports a recent suggestion that H₂ may not be a required intermediate for methanogenesis from formate in M. maripaludis (19), although it may still be needed for biosynthesis. Interestingly, most members of the Methanomicrobiaceae lack genes encoding the F₄₂₀-reducing hydrogenases yet still grow well on H₂; this suggests that there may still be other proteins that interact with Hdr and mediate electron transfer from H₂ to Hdr for reduction of CoMS-S-CoB in these organisms (perhaps the F₂₃₀-reducing hydrogenase) (20).

It should be noted that in addition to providing electrons for Hdr during growth on formate, Fdh must also provide F₂₃₀H₂, which is required for at least one step in methanogenesis (methylene-H₄MPT reductase), and which is also used by F₂₃₀H₂-dependent methylene-H₂MPT dehydrogenase. It has been suggested that F₂₃₀H₂ could also donate electrons to Hdr, based on work in M. voltae that observed an interaction between Hdr and Fru in purified membrane fractions (14). However, deletion of Fru in M. maripaludis had no effect when cells were grown on formate, suggesting that F₂₃₀H₂:hydrodisulfide oxidoreductase activity is not important under these conditions (2).

**Fig. 3.** Model of complex of proteins that interact with Hdr. When H₂ is used as the electron donor for methanogenesis, electrons are transferred to Hdr via Vhu. Flavin-mediated electron bifurcation at Hdr then results in reduction of the CoMS-S-CoB heterodisulfide and a ferredoxin that is used by Fdh for the first step in methanogenesis. When hydrogen is limiting or is replaced by formate, Fdh is highly expressed (10) and incorporates into the complex. When formate is used as the electron donor for methanogenesis, electrons are transferred to Hdr from formate via Fdh. Fd(red), reduced ferredoxin; Fd(ox), oxidized ferredoxin.

**Table 2. Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>Wild type M. maripaludis (34)</td>
</tr>
<tr>
<td>MM901</td>
<td>S2 with an in frame deletion of the uracil phosphoribosyltransferase gene (Mmp0680)</td>
</tr>
<tr>
<td>MM1262</td>
<td>MM901 with an in frame deletion of fhda2B2 (Mmp0138 and Mmp0139)</td>
</tr>
<tr>
<td>MM1263</td>
<td>MM901 with a 6x C-terminal Histidine tag onHdrB1 (Mmp1155)</td>
</tr>
<tr>
<td>MM1264</td>
<td>MM901 with a 6x C-terminal Histidine tag onHdrB2 (Mmp1053)</td>
</tr>
<tr>
<td>MM1265</td>
<td>MM1262 with a 6x C-terminal Histidine tag onFdhA1 (Mmp1298)</td>
</tr>
<tr>
<td>MM1272</td>
<td>MM901 with an in frame deletion of the vhuAU and vhcA regions (Mmp1694, Mmp1693, and Mmp0823)</td>
</tr>
</tbody>
</table>

**Methods**

**Strain Construction.** Strains used in this study are described in Table 2. PCR primers and plasmids can be found in Table S3. MM901 was used as the background strain for all genetic manipulations. To construct MM901, which contains a deletion of the uracil phosphoribosyltransferase gene (Δurp), pBBPrt (4) was digested with MluI-XhoI followed by extension with Klenow fragment and ligated to pBBPrt. This vector was transformed into M. maripaludis S2 as described in ref. 4 and selected in McCas medium (4) containing 1 mg/mL neomycin followed by selection for a mutant containing the in-frame deletion of upp on medium.
containing 250 μg/mL 6-azauracil to resolve the merodiploid. MM901 was transformed with constructs derived from the suicide vector pCRUptNeo to make other markerless gene replacements. pCRUptNeo was constructed exactly as described for the suicide vector pCRPrtNeo (4), except the uppt gene was amplified with Easy-A polymerase (Stratagene) and ligated into the appropriate vectors. To create genomic copies ofHdrB1 orHdrB2 with a C-terminal 6x-His tag, the 3′ region of the gene for HdrB1 or HdrB2 was PCR amplified using Phusion DNA polymerase (Finnzymes) with primers encoding a 10-amino acid extension and blunt-end ligated to a PCR frag-
ment derived from the downstream genomic region of the gene to place the
primer-encoded His-tag at the 3′ end of the ORF. The fragment was ligated to XbaI-Nott digested pCRUptNeo. The resulting vector was transformed into strain MM901 as described (4), with selection of the mutant on McCas plates containing the 250 μg/mL 6-azauracil in place of 8-azaglyoxanthine, to make strains MM1263 and MM1264. A genomic copy of FdhA1 was created with a 13-amino acid extension containing a 6x-His-tag in a deletion strain of
fdh2 locus. First, fdhA2B2 was deleted to generate MM1262 by PCR
amplifying genomic regions flanking the genes with Herculase DNA poly-
merase (Stratagene), digesting the products with Ascl and ligating them
together. This construct was transferred to XbaI-Nott digested pCRUptNeo and transferred into MM901, as described above. The FdhA1 gene was PCR-
amplified and ligated into SpeI-Ascl digested pLCW40neo (11) upstream of a 6x-His tag to make pLCW40fdhA1. The region downstream of fdhA1 in S2
genomic DNA was PCR-amplified and blunt-end ligated to the 3′ end of the
gene encoding the His-tag (PCR amplified from pLCW40fdhA1) and the
construct was transferred into XbaI-Nott digested pCRUptNeo. The resulting
plasmid construct was then transformed as above into strain MM1262 to
generate MM1265. The region encoding the vhvA and vhvu genes was deleted following the same procedure as the deletion of fdh2, except
mutants with reduced growth on H2 were enriched once as described (27) in
McCas medium (4), with 2.5 μg/mL puromycin and a headspace of H2/CO2,
2. Hendrickson EL, Leigh JA (2008) Roles of coregions F500–reducing hydrogenases andhydrogen-
and F250-dependent methylenetetrahydrodiphenol oxygenases in reduction of F250 and production of hydrogen during methanogenesis. J Bacteriol
190:4188–4182.
demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine
Methanococcus maripaludis under defined nutrient conditions. FEMS Microbiol Lett
238:85–91.
6. Xia Q, et al. (2006) Quantitative proteomics of the archaea Methanococcus mari-
paludis validated by microarray analysis and real-time PCR. Mol Cell Proteomics 5:
868–881.
7. Xia Q, et al. (2009) Quantitative proteomics of nutrient limitation in the
hydrogenotrophic methanogen Methanococcus maripaludis. BMC Microbiol 9:149.
binding motif as deduced from studies with subunit B of heterodisulfide redoxase

Mass Spectrometry of Purified Protein Samples. After thawing, 100 μL of
the sample was diluted with 100 μL of 10% acetonitrile in Millipore water
containing sufficient trypsin (Promega) to sequenced (įal) to make the
tryptin-protein ratio = 1:3–1:10. A larger-than-normal quantity of trypsin
was used because of the presence of dithionite in the elution buffer. After
gestation at 37 °C for 12 h, samples were placed in a Speed-Vac (Rota
11749) to bring final volumes to 50 μL. Capillary HPLC/tandem mass spectrometry
was performed in a data-dependent manner using a single dimension sep-
aration with a Michrom Magic 2002 HPLC modified in-house (6) for capillary
columns as described and interfaced to a LTQ linear trap fragmenta-
ter. The samples were loaded on a 10 cm × 75 μm intradimal Aqua C18
reversed-phase capillary column fabricated in-house, flushed 15 min with
Millipore water for desalting, then eluted with a binary gradient as reported
previously for the reversed phase portion of a 2D separation (6, 7). The raw
data files were searched against the Manifold database (6, 7) using Sequest (28) and
peptide level results were organized at the protein level using GroupSeq (29), such that only identifica-
tions were saved, thus allowing a summation of the spectral counts as-
sociated with each protein-encoding ORF in the database. All Sequest and
DTSelect adjustable parameters were set as described (6, 7).

Analysis of Mass Spectral Data. SCs [numbers of peptide detections for a given
protein, i.e., spectral counts (30–32)] have been established as an accurate
method for measuring relative protein abundance in M. maripaludis (7). For
control sample analysis, we sequenced (įal) to make the
tryptin-protein ratio = 1:3–1:10. A larger-than-normal quantity of trypsin
was used because of the presence of dithionite in the elution buffer. After
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DTSelect adjustable parameters were set as described (6, 7).

Analysis of the vhvA UvhvA Mutant During Growth on H2/CO2 or Formate.
MM901 or MM2172 was grown to OD660 ~0.6 in formate medium. Cultures
were washed once with 5 mL N-free medium (33) and
were transferred to tubes containing 5 mL either McCas with a headspace of H2/
CO2 (80:20) or 40 psi or formate medium with 0.2% casamino acids and
CO2 (9:1). The samples were grown for ~24 h to a final OD660 of ~0.4 to 0.5 and collected as described below.

Affinity Purification of Tagged Proteins. Four-hundred milliliters from each
chemostat culture was collected anaerobically as described (11), brought into
an anaerobic chamber (Coy Laboratory Products), and transferred into a 0.5-
L centrifuge bottle. Formate-grown cultures were brought directly into an
anaerobic chamber and transferred to a 0.5-L centrifuge bottle. Samples
were then centrifuged anaerobically at 4 °C at 12,800 × g for 25 min. The
resulting cell pellet was suspended in 1 to 2 mL residual growth medium and
placed in 5 mL glass tubes with an atmosphere of N2/H2 (95:5) and stored
at ~80 °C for up to 2 months. All purifications were done under anaerobic
conditions in an atmosphere of N2/H2 (95:5) in an anaerobic chamber. Cells
were thawed and sonicated on ice using a Microson ultrasonic cell disruptor
190:4188–4182.
demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine
Methanococcus maripaludis under defined nutrient conditions. FEMS Microbiol Lett
238:85–91.
6. Xia Q, et al. (2006) Quantitative proteomics of the archaea Methanococcus mari-
paludis validated by microarray analysis and real-time PCR. Mol Cell Proteomics 5:
868–881.
7. Xia Q, et al. (2009) Quantitative proteomics of nutrient limitation in the
hydrogenotrophic methanogen Methanococcus maripaludis. BMC Microbiol 9:149.
binding motif as deduced from studies with subunit B of heterodisulfide redoxase


17. Wasserfallen A, Nölling J, Costa et al. PNAS.


Disruption of hdrB1 or hdrB2 in MM901, MM1263, or MM1264. Puromycin N-acetyl-transferase (pac) was inserted into the middle of hdrB1 or hdrB2 in MM1264 and MM1263 respectively. Regions for bases 100 to 400 and 400 to 700 in the ORF were PCR-amplified and ligated into pJK3 (1) into restriction sites ~400 bp upstream (ClaI-XhoI) and downstream (NotI-BamHI) of the plasmid encoded pac gene. The construct was linearized with SapI, transformed into MM901, MM1263, or MM1264 as appropriate, and disruptions were selected with medium containing puromycin. MM901 with hdrB1 and hdrB2 disruptions were designated MM1268 and MM1270, respectively. MM1263 and MM1264 with hdrB2 or hdrB1 disrupted were designated MM1271 and MM1269, respectively. Constructs were verified using Southern blots.

Testing the Viability of the His-Tagged Constructs. Strains with pac disruptions of hdrB1 or hdrB2 were grown to OD_{660} ~1.0 in McCas medium with 2.5 μg/mL puromycin and ~0.5 mL was transferred to 5 mL fresh McCas medium with a headspace of H_{2}/CO_{2} (80:20) at 40 psi. MM1262 and MM1265 were grown to OD_{660} ~0.6 in formate medium and ~0.5 mL was transferred to 5 mL fresh formate medium with 0.2% casamino acids and a headspace of N_{2}/CO_{2} (80:20) at 30 psi. Cultures were grown at 37 °C at 100 rpm agitation (Jeio Tech SK-600 shaker). Cell density (OD_{660}) was monitored.

Fig. S2. SDS/PAGE for purifications of the complex from cultures grown with either excess or limiting H₂. Proteins in the size ranges of the purified bands are indicated with their predicted molecular weights in kDa in parentheses. MM901 and MM1262, controls; MM1265, FdhA1-6xHis; MM1263, HdrB1-6xHis; MM1264, HdrB2-6xHis.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOC)
Table S3 (DOC)