Hydrogenases of *Methanococcus maripaludis*

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Objectives

- *The research examines the enzymes and pathways in H_2 metabolism in methanogens, especially as they relate to energy conservation. In addition, the metabolism of formate, from which H_2 is produced, is investigated.*

Technical Barriers

- *The research should lead to an enhanced understanding of how two universal forms of biological energy, the chemical energy contained in the bonds of molecular hydrogen and the energy stored in ion gradients that form across cell membranes, can be interconverted.*

Abstract

The methanogens catalyze a major component of the Earth's H_2 cycle. They are especially active in anaerobic environments where they are the primary consumers of fermentatively produced H_2. These strictly anaerobic Archaea have evolved unique adaptations to H_2 metabolism, many of which are poorly understood. Our research examines the enzymes and pathways in H_2 metabolism in methanogens, especially as they relate to energy conservation (Fig. 1). In addition, the metabolism of formate, from which H_2 is produced, is being investigated. The model species of choice is the hydrogenotroph *Methanococccus maripaludis*. Transcriptome and proteome studies have determined the regulatory effects of H_2 limitation. Studies of protein complexes indicate that the heterodisulfide reductase is membrane-associated and also forms a complex with certain hydrogenases and with formate dehydrogenase. The analysis of mutants has suggested that two alternative pathways function in the interconversion of reduced coenzyme F_{420} and H_2. Finally, growth yield studies with chemostats suggest that two alternative pathways of energy conservation function under differing conditions of H_2 availability.

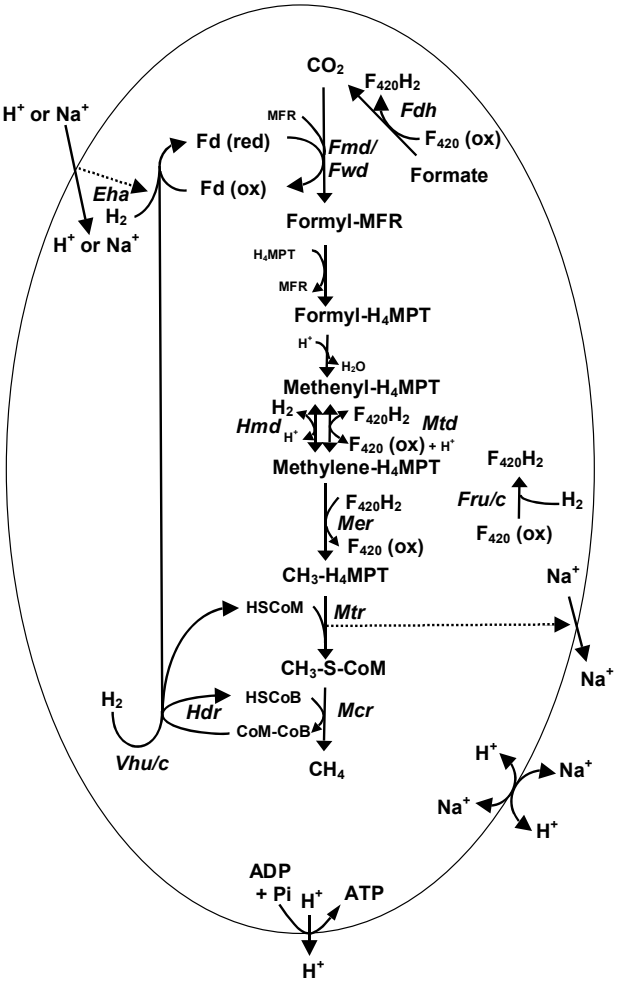

Fig. 1. Methanogenic pathway of the hydrogenotrophic methanogens. Abbreviations for enzymes are in italics: *Eha*, energy-conserving hydrogenase A; *Fdh*, formate dehydrogenase; *Fmd*/*Fwd*, formyl-methanofuran dehydrogenase; *Fru*/*c*, F_{420}-reducing hydrogenase; *Hdr*, heterodisulfide reductase; *Hmd*, H_2-dependent methylene-H_4-methanopterin dehydrogenase; *Mcr*, methyl-coenzyme M reductase; *Mer*, methylene-H_4-methanopterin reductase; *Mtd*, F_{420}-dependent methylene-H_4-methanopterin dehydrogenase; *Mtr*, methyl-H_4-methanopterin-coenzyme M methyltransferase; *Vhu*/*c*, F_{420}-nonreducing hydrogenase. Dotted arrows indicate coupling of metabolic steps with membrane ion gradients.

Progress report

Function of the energy-conserving hydrogenase Ehb and global regulation in an Ehb mutant. We constructed a mutant of *Methanococcus maripaludis* lacking the energy-conserving hydrogenase Ehb and obtained evidence that the mutant was deficient in biosynthetic CO_2 assimilation. Microarrays and differential proteomics confirmed that the mRNA and protein levels for the enzymes of

CO_2 assimilation were elevated in the mutant. These results were consistent with the hypothesis that Ehb coupled the reduction of a low potential ferredoxin with a chemiosmotic membrane gradient. This ferredoxin would then be used to drive the highly endergonic reactions of acetyl-CoA synthase, pyruvate oxidoreductase, and other biosynthetic enzymes that assimilate CO_2.

Identification of genes regulated at the mRNA level by H_2 limitation. Regulation by H_2 availability in methanogens is important because it can identify cellular components that play key roles in H_2 utilization, electron flow, and energy conservation. We devised a system for growth of *M. maripaludis* in chemostats, where growth rate can be controlled and specific nutrient limitations can be imposed. By comparing H_2 limitation to two other nutrient limitations (phosphate limitation and leucine limitation), we were able to discern which genes were regulated by each condition. In addition, we varied growth rate without changing the limiting nutrient, and were able to determine which genes were regulated by growth rate. H_2 limitation markedly increased the mRNA abundance for genes encoding reactions that involve F_{420} as electron acceptor or electron donor. Thus, mRNA levels for *fru*, *mtd*, *mer*, and *fdh* were markedly higher in H_2-limited cultures.

Development of a membrane vesicle system and characterization of the particulate heterodisulfide reductase (pHdr). Inverted membrane vesicles have been indispensable tools in the analysis of chemiosmotic energy coupling, including studies of *Methanosarcina*. We prepared inverted vesicles of *M. maripaludis* by methods developed for *Methanosarcina* strains. The vesicles were assayed for heterodisulfide reductase (Hdr) activity. Prior to these studies, the Hdr from hydrogenotrophic methanogens was reported to be soluble. Because this enzyme was believed to play a fundamental role in coupling methanogenesis to the proton motive force, it was expected to be membrane bound. To examine this point more fully, an assay was developed for the Hdr. For extracts prepared by the French press, 34% of the activity was associated with the membrane fraction. Moreover, the specific activity of washed membranes was 38 nmol min^{-1} mg^{-1}, or comparable to the H_2- or coenzyme F_{420}-dependent activity observed by others. The specific activity of the membranes was also higher than that of the soluble fraction, suggesting enrichment of the Hdr. These results indicate that Hdr activity is membrane-associated and has implications for our model of energy conservation.

Co-purification of possible energy-conserving components with heterodisulfide reductase. We have begun experiments to use the Hdr to "pull down" components of a complex that may be involved in energy conservation. We constructed strains expressing oligo-his tagged $HdrA_c$ and HdrB1, two subunits of heterodisulfide reductases, and conducted anaerobic nickel affinity purifications of cell extracts. We then separated purified proteins by 2-D gel electrophoresis and identified the spots by MALDI-TOF mass spectrometry. Co-purifying proteins included the VhuD subunit of non-F_{420} reducing hydrogenase and the FdhA and FdhB subunits of formate dehydrogenase, as well as additional subunits of Hdr. Note that Fdh is expressed in high levels even in H_2-grown cells. These results are exciting because they suggest that all three enzymes are part of a complex. The complex of Hdr with non-F_{420} reducing hydrogenase has been observed before and is consistent with direct transfer of electrons

from the hydrogenase to Hdr. The complex of Hdr with Fdh is novel and suggests that there may be a direct transfer of electrons from formate to Hdr.

Analysis of membrane proteins by Blue Native PAGE and mass spectrometry. Protein complexes in the membrane fractions of *M. maripaludis* were analyzed using Blue Native PAGE (BN-PAGE) and mass spectrometry. About one-half of the total hydrogenase activity was associated with membrane preparations. Upon BN-PAGE, 16 proteins were identified by in-gel digestion and MALDI-FTICR. Highly expressed cytoplasmic proteins, such as ribosomal and S-layer proteins as well as subunits of the methyl coenzyme M reductase were not detected, indicating efficient separation of the membranes from soluble proteins. The identified proteins included components of three well-known membrane protein complexes, A_1A_0 ATPase, glutamate synthase, and methyl-H_4MPT-HSCoM methyltransferase (Mtr). An additional band contained components of the proteosome. Presumably, this very high molecular weight complex copurified with the membranes. Two bands possessed high levels of hydrogenase activity. Mass spectrometry also identified subunits of the coenzyme F_{420}-reducing hydrogenase (Fru) and energy conserving hydrogenase B (Ehb). The large subunit of coenzyme F_{420}-nonreducing hydrogenase (Vhu) was also identified. Other enzymes co-migrated with these hydrogenases, suggesting that they may form physiologically important complexes. Subunits of the heterodisulfide reductase and formylmethanofuran dehydrogenase co-migrated with the F_{420}-nonreducing hydrogenase Vhu. Similarly, a complex of Vhu and heterodisulfide reductase has also been detected by BN-PAGE of *Methanothermobacter thermoautotrophicus*. The formation of a complex of these three enzymes is consistent with models that couple heterodisulfide reduction to the initial step of CO_2 reduction and the existence of contiguous genes encoding of these proteins. However, this complex is predicted to be soluble and not membrane-bound, as observed here. One band contained the Fru with more components of the formyl-MF dehydrogenase. This seems to provide presumptive evidence for multiple complexes between the formyl-MF dehydrogenase and hydrogenases.

Mutants in F_{420}-reducing hydrogenases (Fru/Frc), F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd), and H_2-dependent methylenetetrahydromethanopterin dehydrogenase (Hmd). One approach we have taken is to make mutants deficient in the hydrogenases and in relevant steps in methanogenesis. We successfully made a strain containing in-frame deletions in the alpha subunits of both F_{420}-reducing hydrogenases, Δ*fruA*-Δ*frcA*. This mutant grew well on both H_2 and formate. Because $F_{420}H_2$ is required for methanogenesis, this finding suggests that there is an alternate pathway for the reduction of F_{420} from H_2. That pathway could be the previously proposed Hmd-Mtd cycle, in which Mtd working in reverse reduces F_{420} (Fig. 1). In addition, we found that during growth on formate, H_2 is produced in the headspace during growth and methanogenesis. The product of formate oxidation is $F_{420}H_2$, and H_2 production evidently occurs from $F_{420}H_2$. There was no decrease in H_2 accumulation in the Δ*fru*-Δ*frc* mutant, suggesting that the Hmd-Mtd cycle can also function to produce H_2. We have also made an *mtd* mutant (Δ*mtd*) and an *hmd* mutant (Δ*hmd*). The only growth defect for either mutant was a lag before growth of the Δ*mtd* mutant on formate. During this lag, the Δ*mtd* mutant accumulated H_2 in the headspace to a higher level than did the other strains. These results are consistent with a complex interaction between Fru/c, Mtd and Hmd. During growth on formate, H_2 is

produced by either the Fru/c or Mtd-Hmd systems, presumably in equilibrium with the formate concentration. However, in the Δ*mtd* mutant, methanogenesis is blocked at the methenyl-H_4MPT step at low levels of H_2. Only when H_2 accumulates to higher levels can the low affinity Hmd become active and methanogenesis continue.

We studied the production of H_2 from formate in resting cells. The characteristics of this process may have implications for the interactions of redox enzymes and whether H_2 is an obligatory intermediate during growth on formate. Deletion of the F_{420}-reducing hydrogenase, Fru, severely decreased H_2 production, suggesting that the major pathway of H_2 production comprised formate dehydrogenase and Fru. Because the Δ*fru* mutant retained 20% of the wild type H_2 production activity, an additional pathway was present. Deletion of the *mtd* or *hmd*, decreased the activity by 20% of the wild-type level, indicating that the second pathway was comprised of formate dehydrogenase-Mtd-Hmd. High rates of formate-dependent H_2 production were consistent with the hypothesis that the H_2 utilization reactions of methanogenesis were reversible and demonstrated the potential of *M. maripaludis* for the microbial production of H_2 from formate.

Growth yields under hydrogen limited chemostat cultures—the uncoupling phenomenon. Hydrogenotrophic methanogens are known to "uncouple" growth from methanogenesis under some circumstances, that is, the cell yield per mol of methane produced or Y_{CH4} is less under some conditions. We used our chemostat system to determine conditions that affect cell yield of *M. maripaludis*. A H_2-limited chemostat produced three to four-fold less methane than a phosphate-limited chemostat. Growth yields (Y_{CH4}, g dry wt of cells per mole CH_4) were 3.18 under H_2 limitation and 0.94 under phosphate limitation. Hence, uncoupling of methanogenesis from growth occurs under phosphate-limited conditions or when H_2 is in excess. This observation indicates that the pathway of electron flow and/or energy coupling varies depending upon the growth conditions. Thauer's recent findings regarding electron flow between the heterodisulfide reductase complex and the formyl-MFR dehydrogenase suggests a biochemical mechanism for the uncoupling. In methanococci, electrons for formyl-MFR dehydrogenase could come predominately from the heterodisulfide reductase complex under H_2-limited conditions, conserving energy, but come predominately from the Eha hydrogenase under H_2 excess, depleting the membrane potential (Fig. 1). While the latter pathway would impose a thermodynamic disadvantage, it could provide a kinetic advantage.

Future directions

We will test the model for energy coupling that is outlined in Fig. 1 above. The coupling of the exergonic heterodisulfide reductase and the endergonic formyl-MFR dehydrogenase reactions are pivotal. We propose that two mechanisms function: the low potential electron donor for formyl-MFR dehydrogenase is generated either by the Eha hydrogenase in a membrane-dependent reaction or by coupling to the Vhu/c-Hdr complex. In addition, we will test a model for electron flow during growth on formate, and determine whether H_2 is a required intermediate.

Publications (including patents) acknowledging the grant or contract

Porat, I., W. Kim, E. L. Hendrickson, Q. Xia, Y. Zhang, T. Wang, F. Taub, B. C. Moore, I. J. Anderson, M. Hackett, J. A. Leigh, and W. B. Whitman. 2006. Disruption of the *ehb* operon limits anabolic CO_2 assimilation in the archaeon *Methanococcus maripaludis*. J. Bacteriol. 188:1373-1380.

Xia, Q., E. L. Hendrickson, Y. Zhang, T. Wang, F. Taub, B. C. Moore, I. Porat , W. B. Whitman, M. Hackett, and J. A. Leigh. 2006. Quantitative proteomics of the archaeon *Methanococcus maripaludis* validated by microarray analysis and real time PCR. Molecular and Cellular Proteomics 5:868-881.

Hendrickson, E. L., Q. Xia, T. Wang, J. A. Leigh, and M. Hackett. 2006. Comparison of spectral counting and metabolic stable isotope labeling for use with quantitative microbial proteomics. Analyst 131:1335-1341.

Xia, Q., E. L. Hendrickson, T. Wang, R. J. Lamont, J. A. Leigh, and M. Hackett. 2007. Protein abundance ratios for global studies of prokaryotes. Proteomics 7: 2904-2919.

Hendrickson, E. L., A. K. Haydock, B. C. Moore, W. B. Whitman, and J. A. Leigh. 2007. Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic Archaea. Proc. Natl. Acad. Sci. USA 104:8930-8934.

Liu, Y., and W.B. Whitman. In press. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. New York Acad. Sci.

Hendrickson, E. L., Y. Liu, G. Rosas-Sandoval, I. Porat, D. Söll, W. B. Whitman, and J. A. Leigh. 2008. Global responses of *Methanococcus maripaludis* to specific nutrient limitations and growth rate. J. Bacteriol. 190:2198-2205.

Hendrickson, E. L., and J. A. Leigh. 2008. Roles of coenzyme F_{420}-reducing hydrogenases and hydrogen- and F_{420}-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F_{420} and production of hydrogen during methanogenesis. J. Bacteriol., submitted.