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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▽

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Reduced coenzyme F_{420} ($F_{420}H_2$) is an essential intermediate in methanogenesis from CO_2. During methanogenesis from H_2 and CO_2, $F_{420}H_2$ is provided by the action of F_{420}-reducing hydrogenases. However, an alternative pathway has been proposed, where H_2-dependent methylenetetrahydromethanopterin dehydrogenase (Hmd) and $F_{420}H_2$-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd) together reduce F_{420} with H_2. Here we report the construction of mutants of *Methanococcus maripaludis* that are defective in each putative pathway. Their analysis demonstrates that either pathway supports growth on H_2 and CO_2. Furthermore, we show that during growth on formate instead of H_2, where $F_{420}H_2$ is a direct product of formate oxidation, H_2 production occurs. H_2 presumably arises from the oxidation of $F_{420}H_2$, and the analysis of the mutants during growth on formate suggests that this too can occur by either pathway. We designate the alternative pathway for the interconversion of H_2 and $F_{420}H_2$ the Hmd-Mtd cycle.

The methanogenic *Archaea* (methanogens) occupy a variety of anaerobic habitats, where they play essential roles in the conversion of hydrogen and other intermediates to methane (10). The hydrogenotrophic methanogens use hydrogen to reduce CO_2 to methane. In addition, some hydrogenotrophs use formate, and a few substitute certain low-molecular-weight alcohols for hydrogen.

The deazaflavin F_{420} is an essential coenzyme of methanogenesis. The reduction of CO_2 to methane requires reduced F_{420} ($F_{420}H_2$), since it is the sole electron donor for the step that reduces methylenetetrahydromethanopterin (methylene-H_4MPT) (Mer in Fig. 1). In addition, $F_{420}H_2$ is the electron donor for $F_{420}H_2$-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd), one of two enzymes that reduce methenyl-H_4MPT. The other enzyme, H_2-dependent methylenetetrahydromethanopterin dehydrogenase (Hmd), uses H_2 directly. mRNA abundance for *mtd* increased markedly under hydrogen-limited growth conditions (4), suggesting that Mtd may be more important when H_2 is limiting.

The F_{420}-reducing hydrogenases (Fru and Frc) reduce F_{420} with H_2. However, an alternative route for this process has been proposed. In *Methanothermobacter marburgensis* the specific activity of F_{420}-reducing hydrogenase, a Ni-Fe hydrogenase, decreased 20-fold under nickel-limited growth conditions. In contrast, the specific activities of Hmd and Mtd, neither of which requires nickel for activity, increased six- and fourfold, respectively (1). These observations led to the proposal that under nickel-limited conditions, F_{420} may be reduced by the concerted action of Hmd and Mtd, the former working in the forward direction (with respect to the methanogenic pathway) and the latter in the reverse direction (1, 2). This pathway is boxed in Fig. 1.

Here we report on the properties of mutants of *Methanococcus maripaludis* that are deficient in Hmd, Mtd, or the F_{420}-reducing hydrogenases. The results demonstrate that neither Hmd nor Mtd is essential, confirming that either enzyme is sufficient for methenyl-H_4MPT reduction. The results also indicate that, in vivo, Hmd and Mtd do indeed constitute an alternate pathway for the reduction of F_{420} with H_2, which we designate the Hmd-Mtd cycle. Furthermore, we show that during growth on formate, H_2 production occurs, evidently by reversal of either the F_{420}-reducing hydrogenase or the Hmd-Mtd cycle.

MATERIALS AND METHODS

Growth of strains and measurement of H_2. *M. maripaludis* was grown on H_2 and CO_2 by standard anaerobic techniques in McCas medium as described elsewhere (6). For growth on formate, McCas medium was modified to contain 200 mM sodium formate and 200 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0), NaCl was decreased to 0.18 M, and the gas atmosphere was 80% N_2 and 20% CO_2 at a pressure of 15 lb/in^2. Cultures (5-ml volume) were inoculated with 0.25 to 0.5 ml of a culture actively growing on formate. Growth was monitored by optical density at 660 nm. The accumulation of H_2 in the headspace (20-ml volume) was measured using a Hach CARLE Series 100 AGC gas chromatograph equipped with a Supelco 60/80 mesh molecular sieve 5A column (6 ft by 1/8 in.) and a trace analytical RGD2 reduction gas detector.

Construction of plasmids and strains. Primers are listed in Table 1, and strains and plasmids are listed in Table 2. PCR products containing the genes *hmd*, *mtd*, *frcA*, and *fruA* and their flanking regions were generated using the primer pairs hmdcln5for and hmdcln5rev, mtdcln5for and mtdcln5rev, frcAfor2 and frcArev2, and fruAfor and fruArev, respectively. The products were cloned into pCR2.1topo to generate phmdtopo, pmtdtopo, pfrcAtopo, and pfruAtopo. An in-frame deletion of *hmd* was produced by PCR of phmdtopo using primers hmddel1 and hmddel2, followed by digestion with AscI and ligation to produce phmddeltopo. pmtddeltopo, pfrcAdeltopo, and pfruAdeltopol were generated in the same way using pmtdtopo and the primers mtddel1 and mtddel3, pfrcAtopo and the primers frcdel1 and frcdel2, and pfruAtopo and the primers frudel1 and

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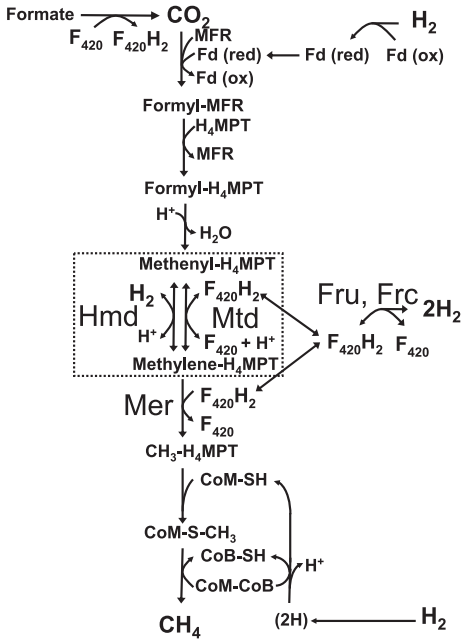

FIG. 1. The hydrogenotrophic methanogenic pathway. See reference 3 for a full description of methanogenesis. The Hmd-Mtd cycle is boxed. Abbreviations: CoB, coenzyme B; CoM, coenzyme M; F_{420}, coenzyme F_{420}; Fd, ferredoxin; Frc, cysteine-containing F_{420}-reducing hydrogenase; Fru, selenocysteine-containing F_{420}-reducing hydrogenase; Mer, methylenetetrahydromethanopterin reductase; MFR, methanofuran.

frudel2, respectively. The in-frame deletion of *hmd* was amplified from phmd-deltopo using the primers hmddelamp1 and hmddelamp3; the resulting fragment was digested with BamHI and ligated into the vector pCRprtneo to produce pCRprtΔhmdneo. pCRprtΔmtdneo was produced in the same way from pmtd-deltopo using the primers mtddelamp1 and mtddelamp2 and digesting with BamHI. pCRprtΔfrcneo was produced from pfrcAdeltopo using frcdelamp5 and frcdelamp6 and digesting with XbaI, and pCRprtΔfruneo was produced from pfruAdeltopo using frudelamp5 and frudelamp6 and digesting with XbaI.

Strains containing markerless in-frame deletions of *hmd*, *mtd*, *frcA*, and *fruA* were constructed in strain Mm900 as described elsewhere (6) using the plasmids pCRprtΔhmdneo, pCRprtΔmtdneo, pCRprtΔfrcneo, and pCRprtΔfruneo, respectively, to produce strains Mm1097, Mm1020, Mm1183, and Mm1145, respectively. A double mutant of *frcA* and *fruA* was constructed by the same procedure from the *frcA* mutant strain Mm1183 by using pCRprtΔfruneo to produce Mm1184. Deletions were confirmed by Southern analysis. For experiments testing whether *hmd* deletion mutations could be made, pCRprtΔhmdneo was transformed into recipient strains. The resultant merodiploids were streak purified, allowed to grow overnight without antibiotic selection, and plated on counterselection plates containing 8-azahypoxanthine. Colonies were analyzed by Southern blotting to distinguish strains containing deletions of the *hmd* gene from those containing the wild-type *hmd* gene.

RESULTS AND DISCUSSION

F_{420} reduction during growth on H_2. We used a genetic approach in *M. maripaludis* to test whether F_{420}-reducing hydrogenase and the Hmd-Mtd cycle constitute two alternative pathways for the reduction of F_{420} in vivo. *M. maripaludis* contains genes for Hmd and Mtd and two sets of genes for F_{420}-reducing hydrogenases, *fruADGB* and *frcADGB* (5). FruA contains selenocysteine residues, while FrcA contains cysteine residues in corresponding positions, and in the closely related *Methanococcus voltae frc* expression is repressed in the presence of selenium in the medium (7, 8). We hypothesized that if Hmd and Mtd can provide an alternative pathway for the reduction of F_{420}, then mutants with deletions in *fru* and *frc* should be viable in the presence of wild-type *hmd* and *mtd*. Conversely, mutants with mutations in either *hmd* or *mtd* should be viable in a *fru*$^+$ *frc*$^+$ background.

Using H_2 and CO_2 as growth substrates, we made the following mutants, all containing markerless in-frame deletions: Δ*fruA*, Δ*frcA*, double mutant Δ*fruA* Δ*frcA*, Δ*mtd*, and Δ*hmd* strains. Δ*fruA* Δ*frcA*, Δ*mtd*, and Δ*hmd* strains each grew normally on H_2 and CO_2 (Fig. 2A). Since $F_{420}H_2$ is essential for methanogenesis, each mutant must retain a pathway for F_{420} reduction using H_2. Hence, the results imply that F_{420}-reducing hydrogenase and the Mtd-Hmd cycle are each sufficient for this function.

As a formal possibility, a third, unknown pathway for the reduction of F_{420}, different from the F_{420}-reducing hydrogenase and the Mtd-Hmd cycle, could exist. To test this possibility, we attempted to construct an Δ*hmd* mutation in a Δ*fruA* Δ*frcA* background. Following our regular procedure for generation of markerless mutations (6), we introduced Δ*hmd* (containing the N- and C-terminal flanking regions of *hmd*) on an integrative vector to produce merodiploids of Δ*hmd* and

TABLE 1. Primers

Name	Sequence	Restriction site
hmdcln5for	GCTGTTGGAATAGACTGCTG	
hmdcln5rev	GCCCTTATTACTTCTTTTCC	
mtdcln5for	CGTTTCAGCAGGTTCGAAGG	
mtdcln5rev	GGGTGTTGCATTAATTGGCG	
frcAfor2	GCACCTCTTTAAAAGCTTT	
frcArev2	AATGAAACAGCGCCATCTAC	
fruAfor	CCAGTACTTCAATATCTTTCAC	
fruArev	TACTTCTTCTGACAACCGAC	
hmddel1	AGGCGCGCCACTTTCATATCAT ACACCTCA	AscI
hmddel2	AGGCGCGCCCAATAAAACCTTA AGTATTAC	AscI
mtddel1	AGGCGCGCCCATTATATCACCG AAAGATAT	AscI
mtddel2	GGGCGCGCCAGAATAAATTTGC ATCAAAAT	AscI
frcdel1	GGCGCGCCTTACCCATCAGATC ACCTATC	AscI
frcdel2	GGCGCGCCAATAAATACTGGTG AATCATGC	AscI
frudel1	GGCGCGCCACTTTATTCACCTCC	AscI
frudel2	GGCGCGCCAATTCTAAATTCCT GAAAAGG	AscI
hmddelamp1	ATGGATCCGGCTTGCTGTTGGA ATAGAC	BamHI
hmddelamp3	TTGGATCCGCCCTTATTACTTCT TTTCC	BamHI
mtddelamp1	GAGCTCGGATCCACTAGTAACG GCCGCCAAGTGT	BamHI
mtddelamp2	AGAATTGGATCCCGTTTCAGCA GGTTCGAAGGA	BamHI
frcdelamp5	ATCTAGAGCACCTTCTTTAAAA GCTTT	XbaI
frcdelamp6	CTCTAGAAATGAAACAGCGCCA TCTAC	XbaI
frudelamp5	TTCTAGACCAGTACTTCAATATC TTTCAC	XbaI
frudelamp6	CTCTAGATACTTCTTCTGACAAC CGAC	XbaI

TABLE 2. Strains and plasmids

Name	Feature(s)	Source or reference
Plasmids		
pCR2.1topo	Amp^r Kan^r cloning vector	Invitrogen
pCRprtneo	*hmv*-promoter-*hpt* fusion + Neo^r cassette in pCR2.1topo	6
phmdtopo	*hmd* plus flanking DNA in pCR2.1topo	This study
pmtdtopo	*mtd* plus flanking DNA in pCR2.1topo	This study
pfrcAtopo	*frcA* plus flanking DNA in pCR2.1topo	This study
pfruAtopo	*fruA* plus flanking DNA in pCR2.1topo	This study
phmddeltopo	In-frame deletion of *hmd* in pCR2.1topo	This study
pmtddeltopo	In-frame deletion of *mtd* in pCR2.1topo	This study
pfrcAdeltopo	In-frame deletion of *frcA* in pCR2.1topo	This study
pfruAdeltopo	In-frame deletion of *fruA* in pCR2.1topo	This study
pCRprtΔhmdneo	In-frame deletion of *hmd* in pCRprtneo	This study
pCRprtΔmtdneo	In-frame deletion of *mtd* in pCRprtneo	This study
pCRprtΔfrcneo	In-frame deletion of *frcA* in pCRprtneo	This study
pCRprtΔfruneo	In-frame deletion of *fruA* in pCRprtneo	This study
Strains		
Mm900	*M. maripaludis* Δ*hpt*	6
Mm1097	Mm900 Δ*hmd*	This study
Mm1020	Mm900 Δ*mtd*	This study
Mm1183	Mm900 Δ*frcA*	This study
Mm1145	Mm900 Δ*fruA*	This study
Mm1184	Mm900 Δ*frcA* Δ*fruA*	This study

hmd$^+$. We made such merodiploids in the Δ*fruA* Δ*frcA*, Δ*frcA*, and *fru*$^+$ *frc*$^+$ backgrounds. We then selected for resolution of the merodiploids via a second recombination event and analyzed the resulting strains by Southern blotting. In principle a mixture of wild-type and deletion strains should result, depending on where the second recombination event occurs. We counted the numbers of resulting Δ*hmd* and *hmd*$^+$ strains in each background. In the *fru*$^+$ *frc*$^+$ background six out of eight strains tested contained Δ*hmd* and the remaining two contained *hmd*$^+$. In the Δ*frcA* background, which should express *fru* and therefore retain active F_{420}-reducing hydrogenase, three strains contained Δ*hmd* and five contained *hmd*$^+$. In contrast, in the Δ*fruA* Δ*frcA* background all 40 strains tested contained only *hmd*$^+$. The results indicate that while Hmd can be eliminated in a strain with active F_{420}-reducing hydrogenase, it is essential in a strain lacking F_{420}-reducing hydrogenase. Therefore, no evidence could be found for the existence of a third pathway that would produce $F_{420}H_2$ from H_2.

H_2 production during growth on formate. Growth on formate differs from growth on H_2 and CO_2 because $F_{420}H_2$ is a direct product of formate oxidation (Fig. 1). Neither the F_{420}-reducing hydrogenase nor the Mtd-Hmd cycle should be necessary for the production of $F_{420}H_2$. However, the reversal of either pathway might result in H_2 production. We characterized the growth of the Δ*fruA* Δ*frcA*, Δ*mtd*, and Δ*hmd* mutants on formate. The Δ*fruA* Δ*frcA* and Δ*hmd* mutants grew normally, while the Δ*mtd* mutant grew after a lag. For each strain, H_2 accumulated in the headspace of the tubes as growth commenced and disappeared when growth ended (Fig. 2B). This observation suggests that H_2 is produced from $F_{420}H_2$ and that either the F_{420}-reducing hydrogenase or the Mtd-Hmd cycle can mediate this conversion. H_2 accumulated to a substantially higher level in tubes containing cultures of the Δ*mtd* mutant than in tubes containing any of the other strains. In the Δ*mtd* strain, Hmd is the only enzyme for the reduction of methenyl-H_4MPT. Therefore, H_2 production, which would occur by the action of the F_{420}-reducing hydrogenase, should be essential. Due to the relatively low affinity of Hmd for H_2 (9), substantially higher H_2 levels accumulate. In contrast, in the other strains Mtd is present and can use $F_{420}H_2$ for the reduction of methenyl-H_4MPT. These results indicate that H_2 production from $F_{420}H_2$ occurs during growth on formate and that either

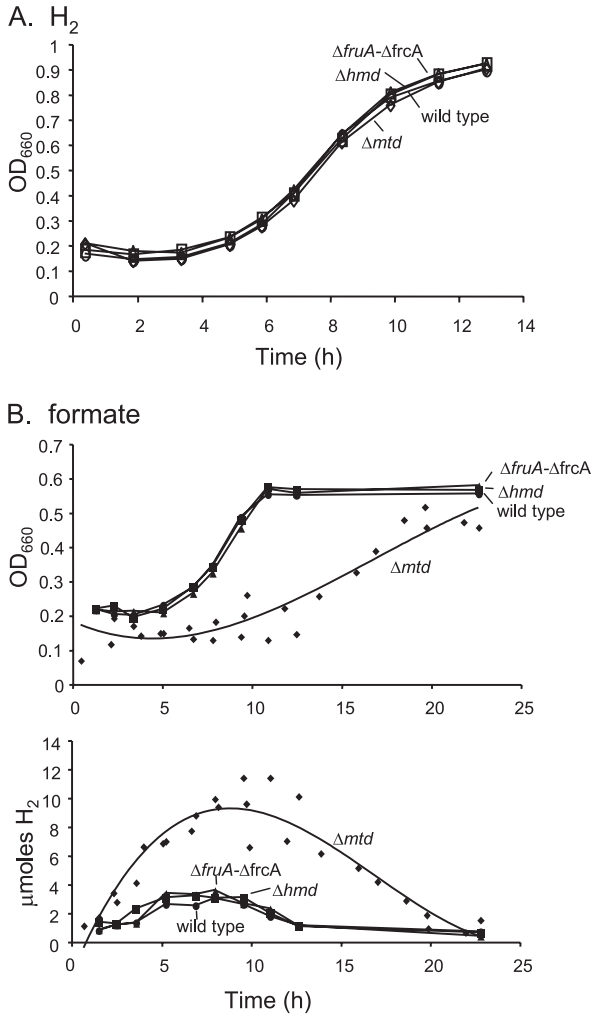

FIG. 2. Growth and H_2 production by wild-type and mutant strains of *M. maripaludis*. (A) Growth on H_2; (B) growth and H_2 production on formate. For the Δ*mtd* mutant on formate, data from three separate growth experiments are plotted and are represented by a single line. OD_{660}, optical density at 660 nm.

the F_{420}-reducing hydrogenase or the Mtd-Hmd cycle can carry out this process.

Whether H_2 is a necessary intermediate during growth on formate cannot be determined from the present data. The generation of a Δ*fruA* Δ*frcA* Δ*hmd* triple mutant, which is expected to grow in the presence of formate, could resolve this question. Growth of the mutant on formate alone without the addition or generation of H_2 would indicate that H_2 is not a required intermediate. A requirement for added H_2 would indicate that H_2 production is required during growth on formate. Efforts to construct such a mutant are under way.

Concluding remarks. The genetic approach taken here has shown that two alternative pathways, the F_{420}-reducing hydrogenase and the Hmd-Mtd cycle, can function in vivo for the reduction of F_{420} with H_2. Furthermore, during growth on formate the same pathways function in reverse to produce H_2 from $F_{420}H_2$. The lack of growth differences between the wild-type and mutant strains on H_2 and CO_2 (Fig. 2A) suggests that neither pathway for F_{420} reduction was rate limiting. However, in nature the F_{420}-reducing hydrogenase may constitute the major pathway when sufficient nickel is present, while the Hmd-Mtd cycle may be important when nickel is limiting (1, 2).

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