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ORIGINAL PAPER

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# Regulation of the synthesis of $H_2$-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) and of HmdII and HmdIII in *Methanothermobacter marburgensis*

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**Abstract** Recently it was found that the specific activity of $H_2$-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) in *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain Marburg) increased six-fold when the hydrogenotrophic archaeon was grown in chemostat culture under nickel-limited conditions. We report here that the increase is due, at least in part, to increased expression of the *hmd* gene. This was demonstrated by Northern and Western blot analysis. These techniques were also used to show that *hmd* expression in growing *M. marburgensis* is not under the control of the $H_2$ concentration. Studies with monoclonal antibodies on the effect of growth conditions on the expression of *hmdII* and *hmdIII*, which have been proposed to encode Hmd isoenzymes, were also carried out. The results indicate that the expression of these two genes is regulated by $H_2$ rather than by nickel, and that HmdII and HmdIII most probably do not exhibit Hmd activity.

**Keywords** Hydrogenase · Methanogenic archaea · Nickel · Transcriptional regulation · *Methanobacterium thermoautotrophicum* · Tetrahydromethanopterin

**Abbreviations** *Hmd* $H_2$-forming methylenetetrahydromethanopterin dehydrogenase · *Mtd* Coenzyme $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase · *Frh* $F_{420}$-reducing hydrogenase · *Mch* Methenyltetrahydromethanopterin cyclohydrolase · *Mer* $F_{420}$-dependent methylenetetrahydromethanopterin reductase · *$H_4$MPT* Tetrahydromethanopterin · *Methenyl-$H_4$MPT$^+$* $N^5$,$N^{10}$-methenyltetrahydromethanopterin · *Methylene-$H_4$MPT* $N^5$,$N^{10}$-methylenetetrahydromethanopterin · *$F_{420}$* Coenzyme $F_{420}$

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## Introduction

$H_2$-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) is an enzyme found in many but not all methanogenic archaea growing on $H_2$ and $CO_2$ as energy source (Thauer et al. 1996). Hmd catalyzes the reversible reduction of $N^5$,$N^{10}$-methenyltetrahydromethanopterin (methenyl-$H_4$MPT$^+$) with $H_2$ to $N^5$,$N^{10}$-methylenetetrahydromethanopterin (methylene-$H_4$MPT) (reaction 1) (Zirngibl et al. 1990; Schleucher et al. 1994).

$$\text{Methenyl-H}_4\text{MPT}^+ + \text{H}_2 \overset{\text{Hmd}}{\rightleftharpoons} \text{methylene-H}_4\text{MPT} + \text{H}^+ \qquad \Delta G^{\circ\prime} = -5.5\ \text{kJ/mol} \quad (1)$$

In all hydrogenotrophic methanogens, reaction 1, which is involved in $CO_2$ reduction to $CH_4$, is also mediated by the combined action of the two enzymes $F_{420}$-reducing hydrogenase (Frh) and $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd) (reactions 2 and 3) (Thauer 1998).

$$\text{F}_{420} + \text{H}_2 \overset{\text{Frh}}{\rightleftharpoons} \text{F}_{420}\text{H}_2 \qquad \Delta G^{\circ\prime} = -11\ \text{kJ/mol} \quad (2)$$

$$\text{Methenyl-H}_4\text{MPT}^+ + \text{F}_{420}\text{H}_2 \overset{\text{Mtd}}{\rightleftharpoons} \text{methylene-H}_4\text{MPT} + \text{F}_{420} + \text{H}^+ \qquad \Delta G^{\circ\prime} = +5.5\ \text{kJ/mol} \quad (3)$$

Frh has a ten-fold lower apparent $K_m$ for $H_2$ than Hmd (Thauer et al. 1996). Based on this finding, it has been proposed that methenyl-$H_4$MPT$^+$ reduction with $H_2$ to methylene-$H_4$MPT is catalyzed by Frh plus Mtd at low $H_2$ concentration and by Hmd at high $H_2$ concentration (Afting et al. 1998). In support of this proposal is the finding that, in *Methanothermobacter thermoautotrophicus* (formerly *Methanobacterium thermoautotrophicum* strain ΔH) (Wasserfallen et al. 2000), the concentration of the

*frh* transcript increases and that of the *hmd* transcript decreases when the $H_2$ supply of the culture is reduced (Morgan et al. 1997; Reeve et al. 1997). Consistent with the proposal are also results of measurements of the specific activity of Frh and Hmd under various growth conditions (Vermeij et al. 1997).

Recently, another function for Hmd has been proposed (Afting et al. 1998). From in vitro studies it was known that, in the presence of methenyl-$H_4MPT^+$, Hmd together with Mtd can catalyze the reduction of $F_{420}$ with $H_2$ (Von Bünau et al. 1991; Klein and Thauer 1995). The two enzymes together thus could substitute for Frh in vivo in catalyzing reaction 2.

Evidence for this has been provided from studies of the specific activity of Hmd, Mtd and Frh in *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain Marburg) (Wasserfallen et al. 2000) grown under nickel-limited conditions: The specific activity of Hmd and Mtd is six- and four-fold, respectively, higher and that for Frh 20-fold lower than in cells grown on nickel-sufficient medium (Afting et al. 1998). For the interpretation of the results, it is important to consider that both Hmd and Mtd are metal-free enzymes, whereas Frh is a nickel-iron-sulfur flavoprotein (Thauer et al. 1996).

The increased specific activity of Hmd and Mtd in cells grown under Ni-limited conditions indicates either that more of the enzymes were synthesized under these conditions or that the enzymes were somehow activated. In the case of Hmd, a third possibility is that the increased specific activity is due to the synthesis of an Hmd isoenzyme. This has to be considered since, in the genome of *M. thermoautotrophicus*, two ORFs, MTH1512 and MTH504, were found. These were predicted to encode proteins with, respectively, 18.8% and 17.6% sequence identity to Hmd (Smith et al. 1997). The encoded proteins, designated HmdII and HmdIII, share 77.4% sequence identity to each other. The three proteins also have very similar molecular masses (Hmd: 37.9 kDa, HmdII: 37.3 kDa, HmdIII: 37.5 kDa) and isoelectric points (Hmd: 4.56, HmdII: 4.82, HmdIII: 4.46). Via Northern blot analysis, evidence has been provided that the *hmdII* and *hmdIII* genes are transcribed and that the transcription of *hmdII* and *hmdIII* is inversely regulated by the $H_2$ concentration. The presence of the HmdII or HmdIII proteins in the cells was not demonstrated (Reeve et al. 1997).

In this report, we provide evidence that the increased specific activity of Hmd in cells of *M. marburgensis* growing under Ni-limited conditions is due, at least in part, to an increased synthesis of the enzyme rather than to an induction of the HmdII and HmdIII proteins. The function of HmdII and HmdIII remains unknown.

## Materials and methods

*Methanothermobacter marburgensis* (DSM 2133) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Coenzyme $F_{420}$ and tetrahydromethanopterin ($H_4MPT$) were purified from *M. marburgensis* as described previously (Breitung et al. 1992). Methylene-$H_4MPT$ was synthesized from $H_4MPT$ and formaldehyde by spontaneous reaction (Escalante-Semerena et al. 1984). The λ ZAP Express gene library was that described by Hochheimer (1997). The cloning vector λ ZAP Express (*Sau*3AI/CIAP treated), the helper phage ExAssist, and *Escherichia coli* strains XLOLR and XL1 Blue MRF′ were from Stratagene. The cloning vector pCR-Blunt was from Invitrogen and the expression vector pET24b+ and *E. coli* BL21 (DE3) were from Novagen.

### Growth of *M. marburgensis* under Ni- or $NH_4^+$-limited conditions

*Methanothermobacter marburgensis* was grown at 65 °C in a 500-ml all-glass chemostat with a culture volume of 360 ml and a dilution rate of 0.079 $h^{-1}$ ($t_d$=9 h). The culture was stirred with a Teflon-coated magnetic paddle at 500 rpm and was gassed with 80% $H_2$/20% $CO_2$/0.2% $H_2S$ at a rate of 400 ml/min via a microliter candle (Porosity 3; Schott, Mainz, Germany). The pH of the culture was 7.0. The medium in the reservoir was composed as described by Schönheit et al. (1980). For Ni limitation, the $NiCl_2$ concentration was lowered from 5 μM to 0.075 μM (with contaminating $Ni^{2+}$ the concentration was 0.2 μM), and for $NH_4^+$ limitation, the $NH_4Cl$ concentration was lowered from 50 mM to 18 mM. Under steady-state conditions, both the Ni-limited and $NH_4^+$ -limited cultures grew at a constant cell concentration of 1.3 g cells (dry mass) per liter, corresponding to a $\Delta OD_{578}$ of 3.2.

To obtain cells grown under the same culture conditions at $\mu_{max}$, non-limited by nickel or ammonium, *M. marburgensis* was grown in the chemostat as batch culture on medium containing 5 μM $NiCl_2$ and 50 μM $NH_4Cl$ and gassed with 80% $H_2$/20% $CO_2$/0.2% $H_2S$. After inoculation (5%), the cells grew exponentially with a doubling time of 2 h to a $\Delta OD_{578}$ of 3.2, at which they were harvested. From the 360-ml culture, approximately 2.3 g cells (wet mass) were obtained.

### Growth of *M. marburgensis* under $H_2$-limited conditions

The growth rate of *M. marburgensis* is dependent on the $H_2$ concentration in the gas mixture with which the cultures are gassed. In batch culture, half-maximal rates were observed at $H_2$ concentrations of 20% $H_2$ (Schönheit et al. 1980). At 5% $H_2$, growth is severely $H_2$-limited. *M. marburgensis* was therefore grown at 65 °C on the mineral salt medium described by Schönheit et al. (1980) in a 2-l all-glass fermenter containing a 1.5 l culture gassed with 5% $H_2$/75% $N_2$/20% $CO_2$/0.2% $H_2S$ at a rate of 800 ml/min and a stirring rate of 900 rpm. The pH of the culture was 7.0. The culture grew with increasing doubling times up to a $\Delta OD_{578}$ of 0.6 and then with a constant doubling time of 11 h up to a $\Delta OD_{578}$ of 1.6. At higher $\Delta OD_{578}$ growth was linear. Therefore, as soon as a $\Delta OD_{578}$ of 1.6 was reached, 750 ml of the culture was transferred to a second 2-l fermenter filled with 750 ml medium. This culture now grew with a doubling time of 11 h directly after inoculation from a $\Delta OD_{578}$ of 0.8 to a $\Delta OD_{578}$ of 1.6. At this $\Delta OD_{578}$, 750 ml of the culture were transferred again and allowed to grow to a $\Delta OD_{578}$ of 1.6. This was repeated another four times before harvesting the cells. By this procedure, cells were obtained that were grown on 5% $H_2$ at a constant doubling time of 11 h for more than five generations.

To obtain cells grown under the same culture conditions near $\mu_{max}$, non-limited by $H_2$, *M. marburgensis* was grown in the fermenter on the same medium gassed with 80% $H_2$/20% $CO_2$/0.2% $H_2S$. The cells grew exponentially from a $\Delta OD_{578}$ of 0.05 to a $\Delta OD_{578}$ of >6 with a doubling time of 1.8 h. The cells were harvested at a $\Delta OD_{578}$ of 1.6. From the 1.5-l culture, approximately 4 g cells (wet mass) were obtained.

### Preparation of cell extracts

Cells were harvested by centrifugation at 4,000×*g* under anoxic conditions. The pellet was then suspended in 8 ml anoxic 50 mM

Tris/HCl, pH 7.6. For cell disruption, the suspension was ultrasonicated at 5 °C three times for 5 min at 60 W with intervals of 5 min. This step was performed in an anaerobic chamber under a gas phase of 5% $H_2$/95% $N_2$. Subsequently, cell debris and membranes were removed by ultracentrifugation under $N_2$ at 75,000×*g* and 4 °C for 30 min. The supernatant is designated cell extract. It could be stored at –20 °C for several days without significant loss of any of the tested enzyme activities.

### Determination of specific enzyme activities

Activities were assayed at 65 °C in 1.5-ml glass cuvettes that contained 0.7 ml assay mixture and $N_2$ or, where indicated, $H_2$ as gas phase; the cuvettes were sealed with a rubber stopper. Anoxic conditions were achieved by repeated evacuation and flushing the cuvettes with the respective gas. Additions were made with microliter syringes. The reactions were started by the addition of cell extract.

Protein was quantified by the method of Bradford (1976) using the BioRad microassay (BioRad Laboratories).

Frh was assayed by following the reduction of $F_{420}$ with $H_2$ photometrically at 401 nm ($\varepsilon_{401}$=25.9 $mM^{-1}$ $cm^{-1}$) (DiMarco et al. 1990). The 0.7-ml assay mixture contained 50 mM Tris/HCl, pH 7.6, 40 μM $F_{420}$ and 2 mM dithiothreitol. The gas phase was 100% $H_2$. One unit (U)=1 μmol $F_{420}$ reduced per min.

Hmd was assayed by following the formation of methenyl-$H_4MPT^+$ from methylene-$H_4MPT$ photometrically at 335 nm ($\varepsilon_{335}$= 21.6 $mM^{-1}$ $cm^{-1}$). The 0.7-ml assay mixture contained 120 mM potassium phosphate, pH 6.0, 40 μM $H_4MPT$, 4 mM formaldehyde and 2 mM dithiothreitol (Zirngibl et al. 1990). The gas phase was 100% $H_2$. One unit=1 μmol substrate transformed per min.

Mtd was assayed by following the formation of methenyl-$H_4MPT^+$ from methylene-$H_4MPT$ photometrically at 335 nm ($\varepsilon_{335}$= 21.6 $mM^{-1}$ $cm^{-1}$). The 0.7-ml assay mixture contained 120 mM potassium phosphate, pH 6.0, 40 μM $H_4MPT$, 4.2 mM formaldehyde, 2 mM dithiothreitol and 40 μM $F_{420}$ (Klein and Thauer 1997). One unit=1 μmol substrate transformed per min. Before the reaction was started with cell extract, the latter was diluted 20-fold with 50 mM potassium phosphate, pH 7.0, containing 2 mM dithiothreitol and was incubated with occasional shaking for at least 2 h at 4 °C. Under these conditions, Hmd in the diluted cell extract was completely inactivated: when $F_{420}$ was omitted from the assay, no methylene-$H_4MPT$ was formed.

$F_{420}$-dependent methylenetetrahydromethanopterin reductase (Mer) was assayed by following the oxidation of $F_{420}H_2$ to $F_{420}$ photometrically at 401 nm ($\varepsilon_{401}$=25.9 $mM^{-1}$ $cm^{-1}$). The 0.7-ml assay mixture contained 50 mM Tris/HCl, pH 7.6, 40 μM $F_{420}$, 2 mM dithiothreitol and 2 mM sodium dithionite for the reduction of $F_{420}$ to $F_{420}H_2$. After incubation for 10 min at 65 °C, 10 mM formaldehyde was added to quench the excess dithionite and 28 nmol $H_4MPT$ was added to generate methylene-$H_4MPT$ from $H_4MPT$ and formaldehyde by spontaneous reaction (Ma and Thauer 1990). One unit=1 μmol $F_{420}H_2$ oxidized per min.

Methylenetetrahydromethanopterin cyclohydrolase (Mch) was assayed by following the hydrolysis of methenyl-$H_4MPT^+$ to $N^5$-formyl-$H_4MPT$ photometrically at 335 nm ($\varepsilon_{335}$=21.6 $mM^{-1}$ $cm^{-1}$). The 0.7-ml assay mixture contained 50 mM Tris/HCl, pH 7.6, 1.5 M potassium phosphate, 30 μM methenyl-$H_4MPT^+$ and 2 mM dithiothreitol.

### Cloning and sequencing of the *hmdII* and *hmdIII* genes

For the study of *hmdII* and *hmdIII* expression in *M. marburgensis*, the two genes from this organism were cloned and sequenced. For the cloning of *hmdII*, a probe (Table 1) was used that was taken from within the *hmdII* gene from *M. thermoautotrophicus* (MTH1512) (Smith et al. 1997). For the cloning of *hmdIII*, a probe (Table 1) from within the *hmdIII* gene from *M. thermoautotrophicus* (MTH504) was used. The probes were labeled with digoxigenin-dUTP and used to screen the λ ZAP Express gene library of *M. marburgensis*. From the positive clones, the phagemids pBK-hmd2–5a and pBK-hmd3–15 were generated by excision and recircularization of pBK-CMV, each carrying a 9-kb *Sau*3A fragment with the *hmdII* and *hmdIII* gene, respectively. The genes were sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit and an ALFexpress sequencer according to the manual of the supplier (Amersham Pharmacia Biotech, Freiburg, Germany). The complete sequences of both strands of the clones were determined and are available at the EMBL Sequence Database under the accession numbers AJ288912 (*hmdII*) and AJ288913 (*hmdIII*). The sequences of the gene products HmdII and HmdIII from *M. marburgensis* show 95.1% and 89.3% identity, respectively, to those of *M. thermoautotrophicus*.

### Heterologous overexpression of *hmdII* and *hmdIII* in *E. coli*

The *hmdII* and *hmdIII* genes were amplified by PCR using pBK-hmd2–5a and pBK-hmd3–15 as DNA template, respectively, and the oligonucleotides 5′-GGCATATGGTGAGCACTATGAAAGTTGC–3′ (sense) and 5′–CCCTCGAGTTCGAACATCTTCCTCATGC–3′ (antisense) for HmdII and 5′-GGCATATGGTGATAACAATGAAGGTG–3′ (sense) and 5′-CCCTCGAGTTCAAACATCTTCC-3′ (antisense) for HmdIII as primers. The sense oligonucleotides were derived from the 5′-sequences of *hmdII* and *hmdIII*, respectively, around the putative start codon GTG and contained a *Nde*I site (restriction site bases underlined). The antisense oligonucleotides were derived from the nucleotide sequence directly upstream of the TAA stop codons and contained a *Xho*I site (restriction site bases underlined). The coding sequence was cloned via PCR-Blunt into the expression vector pET24b+. The sequence now contained a 3′-His-tag-Cassette. With the resulting plasmids pET-hmd2 or pET-hmd3, *E. coli* BL21 (DE3) was transformed. For the expression of *hmdII* or *hmdIII*, *E. coli* was grown

**Table 1** Primers used to generate DNA probes by PCR and oligonucleotides used directly as probes for Northern blot hybridization

| Gene | Primers and oligonucleotides |
|---|---|
| | Primers for probes generated by PCR |
| *hmd* | 5′-GGGCCTGTGAAGTCGCTG-3′ (sense) and 5′-GCAACCGCTTCCTTGTCG-3′ (antisense) |
| *frh* | 5′-GATGTACCGGAGATGCCATGTCGTTAACTG-3′ (sense) and 5′-GTCGCATCCGCATGCCTCTGTGTAGCCTGC-3′ (antisense) |
| | Oligonucleotides used directly as probes |
| *hmdII* | 5′-CCATAAACTGCAACTTTCATAGTGCTCACCTAACATTAATATATTGTTAAGTAATTTGA-3′ |
| *hmdIII* | 5′-TCACCTTCATTGCTATCACTCTTTTCACATTGGAAATCTACAGAAGAAAAGTTTAGTTC-3′ |
| *mtd* | 5′-GGCTGGAACATCAGCCGCTGAAAGGAGCTCCCTTGCCTTTGCAGGGCCAGGTGCACCGG-3′ |
| *mch* | 5′-GAATGGTTTACCGTAGCCCTCAGAGGCTGATGATGGCAGGTTCTCTGCAAGGG-3′ |
| *mer* | 5′-GTAGTGGTCTGTGATCCAGGCGTATTCGAAGCCCACGTCTTCAGCCAGTTTCAC-3′ |

aerobically at 37 °C on 3 l Luria-Bertani medium (Sambrook et al. 1989) containing kanamycin (100 μg/ml) to a $\Delta OD_{578}$ of 0.8 and subsequently induced by addition of isopropylthio-β-D-galactopyranoside to a final concentration of 1 mM. After 4 h further growth, the cells were harvested by centrifugation. From the 3-l culture, approximately 8 g cells (wet mass) were obtained.

### Purification of HmdII and HmdIII from *E. coli*

From the 8 g *E. coli* cells (wet mass), in which *hmdII* or *hmdIII* from *M. marburgensis* were overexpressed, the respective gene product was isolated. The isolation procedure described in the following is that for HmdII and is identical to that for HmdIII. Both proteins contained a C-terminal His-tag and both proteins were almost exclusively recovered in the inclusion body fraction.

The *E. coli* cells were suspended in 50 mM Tris/HCl, pH 7.6, and disrupted by sonication at 4 °C three times for 5 min at 60 W with intervals of 5 min. After centrifugation for 30 min at 10,000×*g* and 4 °C, HmdII was found almost exclusively in the pellet fraction, indicating that it had formed inclusion bodies. The inclusion body fraction was washed once in 50 mM Tris/HCl, pH 7.6, and then dissolved at room temperature in 50 mM Tris/HCl, pH 7.6, containing 6 M urea. After 2 h of stirring, insoluble protein was removed by centrifugation (10,000×*g* for 30 min at 4 °C). Dissolved proteins were loaded on a 1×5-cm $Ni^{2+}$-NTA-agarose column (Qiagen, Hilden, Germany) pre-equilibrated with 50 mM potassium phosphate, pH 7.0, containing 10 mM imidazol, 300 mM NaCl and 6 M urea. HmdII was eluted from the column with the same buffer but containing stepwise-increasing imidazol and NaCl concentrations. HmdII eluted with 50 mM potassium phosphate, pH 7.0, containing 82.5 mM imidazol, 0.725 M NaCl and 6 M urea. Subsequently, HmdII was concentrated and refolded by overnight dialysis against 50 mM potassium phosphate, pH 7.0, using Kollodiumhülsen (Sartorius, Göttingen, Germany).

### Production of rat anti-HmdII and anti-HmdIII monoclonal antibodies

Approximately 50 μg of the fusion proteins were injected intraperitoneally (i.p.) and subcutaneously (s.c.) into LOU/C rats. After a 2-month interval, a final boost was given i.p. and s.c. 3 days before fusion.

Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to standard procedure. Hybridoma supernatants were tested in a solid-phase immuno-assay using HmdII or HmdIII adsorbed to polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound monoclonal antibodies were detected using peroxidase-conjugated goat anti-rat IgG+IgM antibodies (Dianova, Hamburg, Germany) and *o*-phenylenediamine as chromogen in the peroxidase reaction. The immunoglobulin type of the monoclonal antibodies was determined using biotinylated anti-rat IgG subclass-specific monoclonal antibodies (ATCC, Rockville, Mass.). HmdA 8E5 (rat IgG 2b) and HmdB 2C11 (rat IgG 1) reacted selectively in Western blot analysis with HmdII or HmdIII, respectively, and were therefore used in this study.

### Production of polyclonal antibodies against Hmd, Mtd and FrhA

The anti-Hmd serum and the anti-Mtd serum were generated by Eurogentec (Seraing, Belgium) from a rabbit immunized with purified Hmd and Mtd protein, respectively. Anti-FrhA serum was a gift from J. Reeve, Columbus, Ohio.

### Western blot analysis

Polypeptides were separated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Immunodetection was performed using the protocol for the ECL detection kit provided by Amersham Pharmacia with 1:2,500 dilution of rabbit anti-Hmd serum, rabbit anti-FrhA serum or rabbit anti-Mtd serum and 1:10,000 dilution of a peroxidase-conjugated anti-(rabbit-IgG) antibody (BioRad, Munich, Germany) or with 1:10 dilution of rat anti-HmdII monoclonal antibody or rat anti-HmdIII monoclonal antibody and a 1:10,000 dilution of peroxidase-conjugated anti-(rat-IgG) antibody (Dianova). After incubation with the ECL detection reagent, proteins were detected by chemiluminescence according to the manual of the supplier (ECL, Amersham Pharmacia Biotech).

### RNA isolation and Northern blot hybridization

For RNA isolation, 200 ml of *M. marburgensis* culture was cooled to 0 °C in an ethanol/solid $CO_2$ mixture and the cells were harvested by centrifugation at 4,000×*g* in a rotor cooled at 4 °C. After discarding the supernatant, the pellet was transferred into a mortar precooled with liquid nitrogen. The cells were ruptured by grinding in liquid nitrogen for 20 min. RNA was isolated from the ruptured cells using the single-step method for RNA isolation according to Ausubel et al. (1994) or the High Pure RNA Isolation Kit according to the manual of the supplier (Roche Diagnostics, Mannheim, Germany).

The procedure to generate Northern blots was that described by Ausubel et al. (1994). RNA was denatured by glyoxal/dimethylsulfoxide treatment. Hybridization with digoxigenin-labeled DNA probes was performed at 60 °C in 0.75 M NaCl, 75 mM sodium citrate, 0.1% SDS. After an initial wash step at room temperature in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS, the subsequent stringent washing procedure was performed at 68 °C in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS (Hmd, Frh); at 68 °C in 75 mM NaCl, 7.5 mM sodium citrate, 0.1% SDS (Mer, Mtd); at 68 °C in 150 mM NaCl, 15 mM sodium citrate, 0.1% SDS (Mch); or at 60 °C in 225 mM NaCl, 11.25 mM sodium citrate, 0.1% SDS (HmdII, HmdIII).

The digoxigenin-labeled DNA probes for the *hmd* and *frh* mRNA were generated by PCR using primers (Table 1) and the digoxigenin-labeling mixture (Roche, Mannheim, Germany) and those for the *hmdII*, *hmdIII*, *mtd*, *mch* and *mer* mRNA were generated by labeling matching oligonucleotides (Table 1) using the DIG-Oligo-Tailing-Kit.

## Results and discussion

Previous experiments have shown that, in *M. marburgensis*, the specific activity of Hmd and of Mtd increase six- and four-fold, respectively, and that of Frh decreases 20-fold when the organism is grown under Ni-limited conditions (Afting et al. 1998). We now determined by Northern and Western blot analysis whether the regulation is at the level of transcription, translation or posttranslation. As a control, we also determined the expression of the *mer* gene and of the *mch* gene encoding for Mer and Mch, respectively. The specific activity of the two enzymes, which are also involved in $CO_2$ reduction to methane, have been shown not to vary with the growth conditions (Afting et al. 1998).

### Induction of *hmd* and *mtd* and repression of *frh* expression under Ni-limited growth conditions

*Methanothermobacter marburgensis* was grown on 80% $H_2$/20% $CO_2$ under three different conditions: in chemo-

**Table 2** Specific activities and relative abundance of mRNA and protein of four methanogenic enzymes in *M. marburgensis* grown in chemostat culture at a $\Delta OD_{578}$ of 3.2. *Ni-lim* Cells grown in chemostat culture on 80% $H_2$/20% $CO_2$ under nickel-limited conditions ($t_d$=9 h), *$NH_4^+$-lim* cells grown in chemostat culture on 80% $H_2$/20% $CO_2$ under $NH_4^+$-limited conditions ($t_d$=9 h), *non-lim* cells grown at $\mu_{max}$ on 80% $H_2$/20% $CO_2$ in batch culture under non-limited conditions ($t_d$=2 h), – not detectable, +, ++, +++ and ++++ very low, low, medium and high abundance, *n.d.* not determined. The specific activities are mean values from ten measurements in three independent experiments. The deviations were smaller than ±15%

| Methanogenic enzymes | Specific activity (U/mg) | | | mRNA determined by Northern analysis | | | Enzyme protein determined by Western analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ni-lim | $NH_4^+$-lim | non-lim | Ni-lim | $NH_4^+$-lim | non-lim | Ni-lim | $NH_4^+$-lim | non-lim |
| Hmd | 64.7 | 9.5 | 11.1 | ++++ | ++ | ++ | ++++ | + | + |
| Mtd | 21.3 | 4.1 | 5.3 | +++ | ++ | ++ | +++ | + | + |
| Frh | 0.005 | 0.9 | 1.4 | – | ++ | ++ | – | + | + |
| Mer | 7.9 | 7.3 | 6.9 | +++ | +++ | +++ | n.d. | n.d. | n.d. |
| Hmd II | n.d. | n.d. | n.d. | +++ | +++ | +++ | +++ [a] | +++ [a] | +++ [a] |
| Hmd III | n.d. | n.d. | n.d. | – | – | – | ++++[b] | ++++[b] | ++++[b] |

[a]Approximately 2 μg HmdII per mg cell extract protein
[b]Approximately 0.5 μg HmdIII per mg cell extract protein

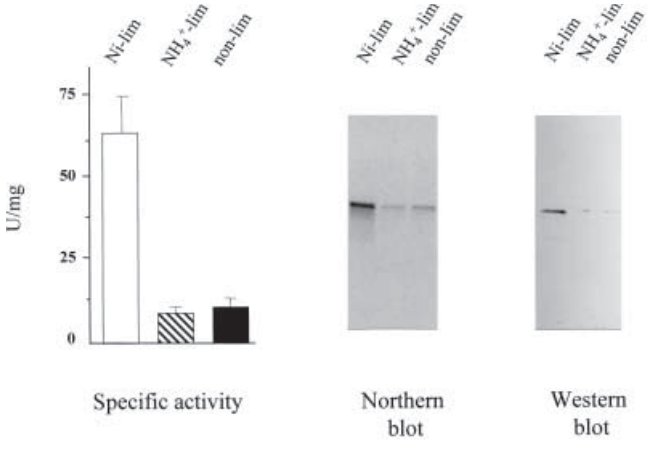


**Fig. 1** Effect of Ni limitation on the expression of the *hmd* gene in growing *Methanothermobacter marburgensis* cells. The cells were grown in chemostat culture at a $\Delta OD_{578}$ of 3.2, harvested and then analyzed for specific activity, Hmd mRNA and Hmd protein. *Ni-lim* Cells grown in chemostat culture on 80% $H_2$/20% $CO_2$ under nickel-limited conditions ($t_d$=9 h), *$NH_4^+$-lim* cells grown in chemostat culture on 80% $H_2$/20% $CO_2$ under $NH_4^+$-limited conditions ($t_d$=9 h), *non-lim* cells grown at $\mu_{max}$ in batch culture on 80% $H_2$/20% $CO_2$ under non-limited conditions ($t_d$=2 h)

stat culture limited by either the nickel concentration (0.2 μM) or the $NH_4^+$ concentration (18 mM) and in batch culture. Under steady-state conditions, the two chemostat cultures had a $\Delta OD_{578}$ of 3.2 and grew with a $t_d$ of 9 h. The batch cultures grew with a doubling time of 2 h and, like the chemostat culture, were harvested at a $\Delta OD_{578}$ of 3.2. The harvested cells were then analyzed for Hmd, Mtd, Frh and Mer specific activity, mRNA and enzyme protein. The results are summarized in Table 2.

The results shown in Table 2 indicate that the high specific activity of Hmd and Mtd in cells grown under Ni-limited conditions is due to an increased concentration of these two enzymes and the low specific activity of Frh to a decreased concentration of the hydrogenase. The relative abundance of the mRNA indicates that the synthesis of the three proteins is, at least in part, regulated at the transcriptional level. A further regulation at the translational level or the level of protein activation cannot be completely ruled out. Figure 1 shows the Northern and Western blots on which the data given for Hmd in Table 2 are based.

## Insignificant effect of $H_2$ on *hmd* expression

It has been proposed that, in *M. marburgensis* growing under $H_2$-limited conditions, molecular hydrogen is activated by Frh, and under $H_2$-non-limited conditions by Hmd. To test this we grew *M. marburgensis* in batch culture on 80% $H_2$/20% $CO_2$ and on 5% $H_2$/75% $N_2$/20% $CO_2$ for at least five generations. The culture on 80% $H_2$ grew exponentially with a doubling time of 2 h whereas the culture with 5% $H_2$ grew exponentially with a doubling time of 11 h. Clearly, growth of the cultures with 5% $H_2$ was $H_2$-limited. The cultures were harvested at a $\Delta OD_{578}$ of 1.6 and analyzed for Hmd-, Mtd-, Frh-, Mer- and Mch-specific activity, mRNA and enzyme protein. In the cells grown under $H_2$-limited conditions, the specific activity of Hmd was always very similar to that in cells grown with 80% $H_2$. Also, the Northern and Western blots did not show significant differences in transcription and translation (Fig. 2). The specific activity, however, of Mtd was two- to three-fold higher in cells grown on 5% $H_2$ than in those grown on 80% $H_2$. Northern and Western blot analysis revealed that the concentration of Mtd mRNA and protein was also higher in cells grown under $H_2$-limited conditions.

Under nickel-limited conditions, Frh synthesis was repressed, whereas that of Hmd was induced (Table 2). Under $H_2$-limited conditions, Frh synthesis was induced, whereas Hmd synthesis remained constant (Table 3). These findings indicate that *hmd* and *frh* expression is not always inversely regulated.

Morgan et al. (1997) described that the *hmd* transcript in *M. thermoautotrophicus* disappeared when growth of

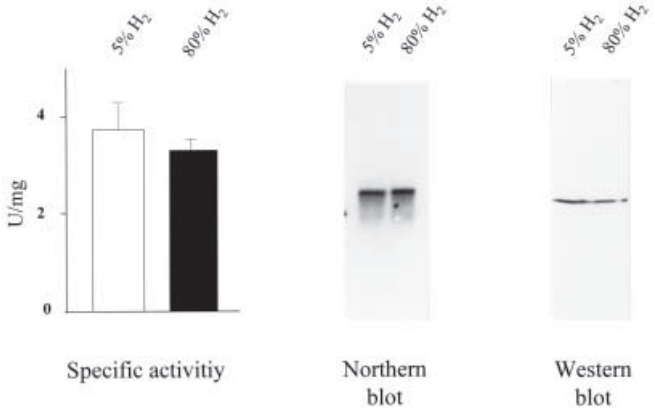


**Fig. 2** Effect of $H_2$ limitation on the expression of the *hmd* gene in growing *M. marburgensis*. The cells were grown in batch culture on 80% $H_2$/20% $CO_2$ ($t_d$=2 h) or 5% $H_2$/75% $N_2$/20% $CO_2$ ($t_d$=11 h) to a $\Delta OD_{578}$ of 1.6, harvested and then analyzed for specific activity, Hmd mRNA and Hmd protein

the organism on 80% $H_2$/20% $CO_2$ was almost stopped, after reduction of the mixing rate in the fermenter from 600 rpm to 120 rpm. The *hmd* transcript appeared and growth resumed again after returning the impeller speed to 600 rpm. The results were interpreted to indicate that *hmd* expression is regulated by $H_2$ supply. The results cannot, however, be directly compared with ours since they were obtained in a down-shift experiment, whereas the constant concentration of the *hmd* transcript observed by us in cells grown under $H_2$ limitation was found under steady-state conditions. Another difference could be that *M. thermoautotrophicus* differs from *M. marburgensis* in $H_2$ regulation. That this could be the case is indicated by the finding that *M. thermoautotrophicus* grows exponentially on 80% $H_2$/20% $CO_2$ only up to a $\Delta OD_{578}$ of 2.5, whereas under the same conditions *M. marburgensis* continues exponential growth up to a $\Delta OD_{578}$ of 6 and higher (Brandis et al. 1981).

## Inverse regulation of HmdII and HmdIII synthesis by $H_2$

The cells of *M. marburgensis* grown under Ni-limited conditions (Table 2) and of those grown under $H_2$-limited conditions (Table 3) were also analyzed for HmdII and HmdIII mRNA and protein (Fig. 3). The results indicate that nickel limitation did not affect either the relative abundance of HmdII and HmdIII mRNA or the concentration of these two proteins. By contrast, $H_2$ limitation had a strong effect on the expression of the *hmdII* and *hmdIII* genes: the relative abundance of the HmdII mRNA and protein was much higher in cells grown with 5% $H_2$ than in the 80%-$H_2$-grown cells or in the cells grown under Ni- or $NH_4^+$-limited conditions (Table 2). In the 5%-$H_2$-grown cells, neither HmdIII mRNA nor HmdIII protein could be detected. For reasons not understood, HmdIII mRNA was also not detectable in all the other cells tested. The absence of the HmdIII protein, however, is indicative, since this protein was present in all the other analyzed cells.

By quantitative Western blot analysis it was found that extracts of cells grown on 80% $H_2$/20% $CO_2$ contain approximately 2 μg HmdII per mg protein (Table 2) and approximately 0.5 μg HmdIII per mg protein. However qualitative Western blot analysis showed that the relative abundance of HmdIII was higher than that of HmdII (Table 2). This apparent discrepancy is due to differences in affinity of the monoclonal antibodies used.

## Attempts to completely separate HmdII and HmdIII from Hmd

Hmd, HmdII and HmdIII have very similar molecular masses and isoelectric points, indicating that these proteins are difficult to separate – and this was indeed the case. It was possible to obtain Hmd preparations that were completely devoid of HmdII and HmdIII, but not HmdII and HmdIII preparations that were devoid of Hmd, as re-

**Table 3** Specific activities and relative abundance of the mRNA and the protein of four methanogenic enzymes in *M. marburgensis* grown in batch culture on 80% $H_2$/20% $CO_2$ ($t_d$=2 h) or 5%$H_2$/75% $N_2$/20% $CO_2$ ($t_d$=11 h). The cells were harvested at a $\Delta OD_{578}$ of 1.6. – Not detectable, +, ++, +++, ++++ very low, low, medium and high abundance, *n.d.* not determined. The specific activities are mean values from 10 measurements in three independent experiments. The deviations were smaller than ±15%

| Methanogenic enzymes | Specific activity (U/mg) | | mRNA determined by Northern analysis | | Enzyme protein determined by Western analysis | |
|---|---|---|---|---|---|---|
| | 5% $H_2$ | 80% $H_2$ | 5% $H_2$ | 80% $H_2$ | 5% $H_2$ | 80% $H_2$ |
| Hmd | 3.9 | 3.3 | +++ | +++ | ++++ | +++ |
| Mtd | 8.0 | 3.7 | ++++ | ++ | ++++ | ++ |
| Frh | 1.2 | 0.6 | ++++ | ++ | +++ | + |
| Mer | 2.5 | 1.0 | ++++ | ++ | n.d. | n.d. |
| Mch | 10.1 | 10.1 | +++ | +++ | n.d. | n.d. |
| Hmd II | n.d. | n.d. | +++ | + | ++++[a] | +[b] |
| Hmd III | n.d. | n.d. | – | – | – | ++++[c] |

[a]Approximately 8 μg HmdII per mg cell extract protein
[b]Approximately 2 μg HmdII per mg cell extract protein
[c]Approximately 0.5 μg HmdIII per mg cell extract protein

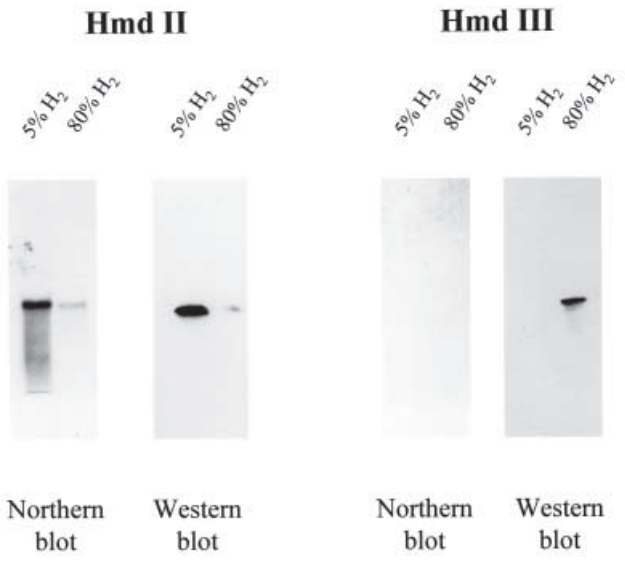


**Fig. 3** Effect of $H_2$ limitation on the expression of the *hmdII* and *hmdIII* genes in growing *M. marburgensis*. The cells were grown in batch culture on 80% $H_2$/20% $CO_2$ ($t_d$=2 h) or 5% $H_2$/75% $N_2$/20% $CO_2$ ($t_d$=11 h) to a $\Delta OD_{578}$ of 1.6, harvested and then analyzed for HmdII and HmdIII mRNA and for HmdII and HmdIII proteins

vealed by Western blot analysis. An elution profile of the three proteins from the Q-Sepharose column eluted with a NaCl gradient is shown in Fig. 4. As can be seen, Hmd actvity was found in all the fractions containing HmdII and HmdIII. Not shown is that in all fractions small amounts of Hmd protein were found by Western blot analysis. All attempts to separate Hmd protein and activity completely from HmdII and HmdIII using other chromatographic steps failed. It was therefore not possible to completely exclude that HmdII and/or HmdIII exhibit some Hmd activity.

## Function of HmdII and HmdIII

If HmdII and HmdIII do not exhibit Hmd activity, what else could their function be? The finding that transcription of *hmdII* is up-regulated and that of *hmdIII* down-regulated under $H_2$-limited growth conditions indicates a direct or indirect role in $H_2$ metabolism. The purified enzymes were therefore tested for hydrogenase activity with $F_{420}$, methylenetetrahydromethanopterin, NAD(P) and viologen dyes as electron acceptors and also for $F_{420}H_2$:NADP oxidoreductase activity. The latter enzyme is involved, together with Frh, in NADP reduction with $H_2$ and is the only other enzyme in *Methanothermobacter* that shows some sequence similarity to HmdII (17.3%) and HmdIII (16.8%). None of the tested activities were found.

In *Ralstonia eutropha* growing on $H_2$ and $O_2$, a nickel iron-sulfur protein was found that senses the $H_2$ concentration and that is involved in a multi-component regulatory system controlling the transcription of $H_2$-regulated genes (Lenz and Friedrich 1998; Pierik et al. 1998). It is not likely that HmdII and HmdIII have a similar function since both proteins lack a nickel binding site characteristic for [NiFe] hydrogenases or sequence motifs characteristic for Fe/S clusters. They both contain only three conserved cysteines.

## Analysis of the intergenic regions

The intergenic regions upstream of the seven genes *hmd*, *mtd*, *frh*, *mch*, *mer*, *hmdII* and *hmdIII* were analyzed for archaeal promotor elements (Soppa 1999a, b); nothing unusual was found. We also looked by manual alignment and visual inspection for additional conspicuous sequence motifs that could bind regulatory proteins. It was hoped that such motifs would be found in the regions upstream of *hmd* and *mtd*, since transcription of both of these genes

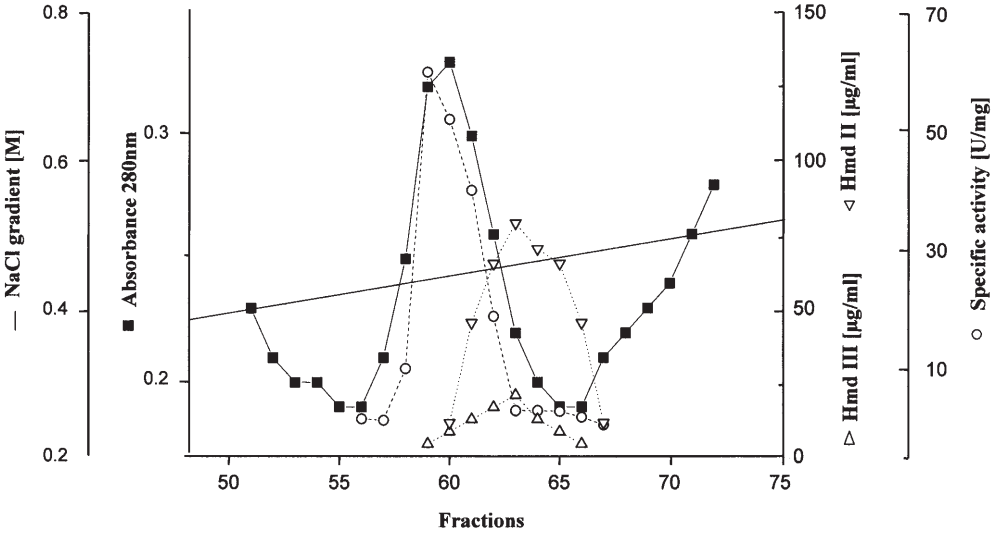


**Fig. 4** Elution profiles of Hmd (○) HmdII (△) and HmdIII (▽) from a Q-Sepharose column loaded with cell extract of *M. marburgensis* grown in batch culture on 80% $H_2$/20% $CO_2$ to a $\Delta OD_{578}$ of 4.0 ($t_d$=2 h). Protein was precipitated with 25 mM $ZnCl_2$, centrifuged (30 min at 10,000×*g*) and the supernatant was loaded onto the 2.6×10-cm (53.4 ml) column. The column was pre-equilibrated with 50 mM MOPS/KOH, pH 7.0, and eluted with a gradient from 0 to 0.65 M NaCl in 50 mM MOPS/KOH, pH 7.0. Fractions of 8 ml were collected. Hmd was detected by its activity and HmdII and HmdIII proteins were detected by Western blot analysis

was found to be up-regulated more than five-fold under nickel-limited conditions (Table 2). A characteristic sequence motif for the binding of a metal-dependent repressor, as recently found in *Archaeoglobus fulgidus* (Bell et al. 1999), could, however, not be identified. This is of interest since the repressor in *A. fulgidus* has been shown to be active with nickel and since two homologues of the repressor are present in *M. thermoautotrophicus*, as predicted in the genome sequence (Smith et al. 1997).

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