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The metal-free hydrogenase from methanogenic archaea: evidence for a bound cofactor

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Abstract The *hmd* gene, which encodes the metal-free hydrogenase in methanogenic archaea, was heterologously expressed in *Escherichia coli*. The overproduced enzyme was completely inactive. High activity could, however, be induced by the addition of ultrafiltrate from active enzyme denatured in 8 M urea. The active fraction in the ultrafiltrate was heat-labile and migrated on gel filtration columns with an apparent molecular mass well below 1000 Da.

Key words: Hydrogenase; H_2-forming methylenetetrahydromethanopterin dehydrogenase; Methanogenic archaea; *Methanobacterium thermoautotrophicum*; *Methanococcus jannaschii*; *Methanopyrus kandleri*

1. Introduction

Many methanogenic archaea growing on H_2 and CO_2 contain an unusual hydrogenase without nickel or iron–sulfur clusters [1,2], which was designated metal-free hydrogenase in order to distinguish it from all other hydrogenases that are Fe/S or Ni/Fe/S proteins [3,4]. The metal-free hydrogenase, abbreviated Hmd, catalyzes the reversible reduction of methenyltetrahydromethanopterin with H_2 to methylenetetrahydromethanopterin ($\Delta G^{\circ\prime} = -5.5$ kJ/mol) which is an intermediate reaction in the pathway of methane formation from CO_2 and H_2 [5,6]. Hmd is composed of only one type of subunit with an apparent molecular mass of approximately 40 kDa and has a specific activity of above 1000 U/mg [7]. The metal-free hydrogenase exhibits a ternary complex rather than a ping-pong kinetic mechanism and it per se does not catalyze a H_2/H^+ exchange or the conversion of *para* H_2 to *ortho* H_2 [2]. These and other findings are consistent with a catalytic mechanism in which the substrate methenyltetrahydromethanopterin is converted by the enzyme to a carbocation which directly reacts with H_2 [8,9]. The mechanism is supported by ab initio molecular orbital calculations [10–12].

Purified active Hmd exhibits an almost pure protein UV/visible spectrum. Only the absorbance at 280 nm was found to be 2.7 times higher than the absorbance calculated from the tyrosine and tryptophan content of Hmd [7]. After denaturation of the enzyme in guanidinium chloride and gel filtration the absorbance was exactly that predicted from the amino acid composition. It was therefore concluded "that the enzyme could contain a prosthetic group with an absorption maximum near 280 nm" [7]. We now provide evidence for the presence of a tightly bound cofactor.

Hmd is a labile enzyme rapidly inactivated in the presence of O_2 [1]. So far only the enzyme from thermophilic methanogens is relatively stable under anaerobic conditions and easy to purify [7,13]. The following experiments were therefore performed with Hmd from *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain Marburg) [14], from *Methanococcus jannaschii* and from *Methanopyrus kandleri*. These hydrogenotrophic methanogens have temperature optima for growth of 65, 85 and 98°C, respectively. The *hmd* genes from the three thermophiles have been cloned and sequenced [2,7,15,16].

2. Materials and methods

Tetrahydromethanopterin (H_4MPT) and methenyl-H_4MPT$^+$ were purified from *M. marburgensis* [17]. Methylene-H_4MPT was prepared from H_4MPT and formaldehyde by spontaneous reaction [18]. Active H_2-forming methylene-H_4MPT dehydrogenase (Hmd) was purified from *M. marburgensis* [1], from *M. jannaschii* and from *M. kandleri* [13] with specific activities of 1500, 350 and 600 U/mg, respectively. *M. marburgensis* was grown in batch culture under nickel-limited conditions [6,19]. The cells of *M. jannaschii* and *M. kandleri* were a gift of K.O. Stetter (Regensburg).

H_4MPT rapidly autoxidizes and Hmd, as mentioned above, is inactivated in the presence of O_2. Therefore, activity assays and enzyme purifications were performed under strictly anaerobic conditions, where possible in an anaerobic glove box (Coy) filled with 95% N_2/5% H_2 and containing palladium catalyst for the continuous removal of O_2. Protein concentrations were determined according to the method of Bradford [20] using the reagents of Bio-Rad Laboratories. Bovine serum albumin was used as standard.

2.1. Determination of Hmd activity

The assays were performed at 65°C in 1 ml quartz cuvettes closed with rubber stoppers [1]. The 0.7 ml assay mixture for the determination of methylene-H_4MPT dehydrogenation contained: 120 mM potassium phosphate pH 6.0; 40 μM H_4MPT; 2 mM formaldehyde (methylene-H_4MPT is formed spontaneously from H_4MPT and form-

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Abbreviations: Hmd, H_2-forming methylenetetrahydromethanopterin dehydrogenase or metal-free hydrogenase; H_4MPT, tetrahydromethanopterin; Fmoc, fluoren-9-ylmethoxycarbonyl

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aldeyde); Hmd in the amounts indicated; and ultrafiltrate of denatured Hmd from *M. marburgensis* in the amounts indicated. The gas phase was 100% N_2. Except when otherwise stated, the reaction was started by the addition of H_4MPT and followed by measuring the formation of methenyl-H_4MPT$^+$ at 336 nm ($\varepsilon_{336} = 21.6$ mM^{-1} cm^{-1}). One unit enzyme activity refers to 1 μmol methylene-H_4MPT converted to methenyl-H_4MPT$^+$ per minute.

The 0.7 ml assay mixture for the determination of methenyl-H_4MPT$^+$ reduction with H_2 contained: 120 mM potassium phosphate pH 7.5; 40 μM methenyl-H_4MPT$^+$; and Hmd and ultrafiltrate in the amounts indicated. The gas phase was 100% H_2. The reaction was started by the addition of ultrafiltrate and followed by measuring the consumption of methenyl-H_4MPT$^+$ at 336 nm. One unit = 1 μmol/min.

2.2. Preparation of ultrafiltrate from denatured Hmd

Hmd (0.7 mg) from *M. marburgensis* with a specific activity of 1500 U/mg was added under strictly anaerobic conditions to 1 ml of saturated urea solution containing 20 mM potassium phosphate pH 7.8 and 20 mM β-mercaptoethanol. After 10 h at room temperature the solution was ultrafiltered in a Centricon filter device (Millipore) with a 10 kDa molecular mass cut-off membrane. Via this procedure 0.8 ml ultrafiltrate of denatured Hmd were obtained and tested in enzyme assays. When due to high activity less than 2.5 μl of ultrafiltrate had to be added to the enzyme assays the ultrafiltrate was diluted in 20 mM potassium phosphate pH 7.8. The volumes given in the text and figures are those of the undiluted ultrafiltrate. Urea-freed ultrafiltrate was obtained by passing 0.25 ml of the ultrafiltrate through a Superdex 200 HR 10/30 column (average particle size 13 μm) from Pharmacia equilibrated and eluted with H_2O. The flow rate was 0.5 ml/min.

2.3. Heterologous expression of the hmd gene in Escherichia coli

The *hmd* gene MJ0784 from *M. jannaschii* [16] was amplified by polymerase chain reaction (PCR) using purified genomic DNA as template. Sense and antisense primers, that were used, contained a *Nde*I and *Bam*HI restriction site, respectively. The amplified DNA fragment was afterwards cloned into the cloning vector pCR Blunt (Invitrogen) and then into the expression vector pET 24b (Novagen) with which *E. coli* HMS 174 (DE) was transformed. The transformed cells were grown at 37°C under strictly anaerobic conditions in 2 l M9 mineral salt medium [21] containing 20 mM glucose and 20 mM potassium nitrate. At an OD_{578} of 0.9 Hmd production was induced by the addition of isopropylthiogalactoside (0.1 mM). Three h after induction the cells (4–5 g wet mass) were harvested by centrifugation.

Cloning and heterologous expression of the *hmd* gene from *M. kandleri* [7] in *E. coli* were performed correspondingly with the exception that the expression vector pET 11a was used instead of pET 24b (both from Novagen).

2.4. Purification of heterologously produced Hmd

E. coli cells (4–5 g wet mass) containing heterologously produced Hmd from *M. jannaschii* were anaerobically suspended in 15 ml 20 mM potassium phosphate pH 7.0 and then disrupted by 80 W ultrasonication for 3×6 min at 0°C. Cell debris were removed by ultracentrifugation at 116 000×*g* for 45 min yielding the cell extract. From the cell extract, which did not exhibit Hmd activity, the protein was purified under strictly anaerobic conditions using a heat step (15 min, 70°C) and size exclusion chromatography with a Superdex 200 HR 10/30 column from Pharmacia. The column was equilibrated and eluted at room temperature with 20 mM potassium phosphate pH 7.0 containing 300 mM NaCl. After concentrating and desalting, the Hmd solution (20 mg/ml) was stored anaerobically in aliquots of 10 μl at −80°C. From 4–5 g cells (wet mass) approximately 10 mg heterologously produced Hmd from *M. jannaschii* were obtained. Via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) the preparation was estimated to be more than 70% pure. Major contaminants were a protein with an apparent molecular mass of 18 kDa and one with an apparent molecular mass of 12 kDa.

The heterologously produced inactive Hmd from *M. kandleri* was purified to apparent homogeneity by ammonium sulfate fractionation (50–60%), by ion exchange chromatography using a Q-Sepharose column HiLoad 26/10 (particle size 24–44 μm) and a 6 ml Resource Q column (average particle size 15 μm) and by gel filtration with a Superdex 200 HR 10/30 column (all from Pharmacia).

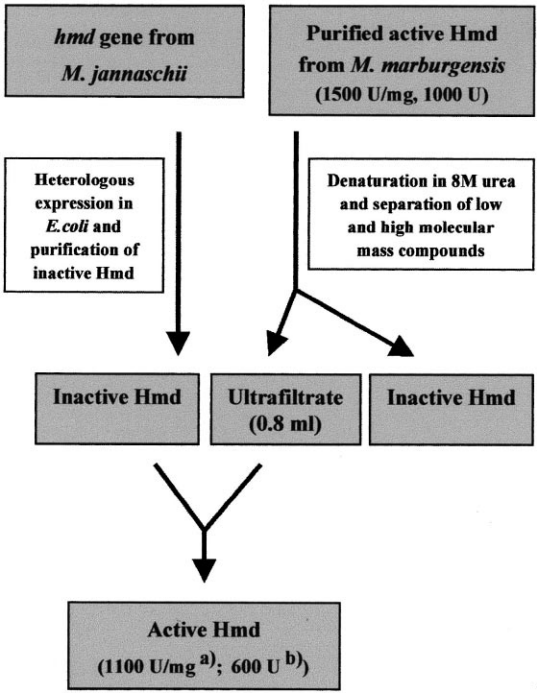

Fig. 1. Scheme of the experiment showing that inactive Hmd heterologously produced in *E. coli* can be activated by ultrafiltrate from active Hmd denatured in 8 M urea. a: Activity determined at saturating concentrations of ultrafiltrate (see Fig. 3A). b: Activity determined at saturating concentrations of heterologously produced Hmd (see Fig. 3B).

3. Results and discussion

In the following it is described that Hmd purified from methanogens can be unfolded in urea and refolded again to the active enzyme. This property does not correspond to the finding that heterologously produced Hmd was always inactive even when unfolded and refolded under the same conditions. This apparent discrepancy led to the discovery that active Hmd contains a tightly bound cofactor.

3.1. Denaturation of Hmd in urea and conditions of renaturation to the active enzyme

Hmd from *M. marburgensis* (0.7 mg; 1000 U) was rapidly inactivated in 1 ml 8 M urea pH 7.0 containing 1 mM EDTA. Approximately 90% of the activity was lost within 2 min at room temperature. Up to 50% of the original Hmd activity could be recovered when after 2–3 h of denaturation the urea was removed by dialysis for 3–4 h in 1 ml collodion bags (Sartorius) against 20 mM potassium phosphate pH 7.0 containing 1 mM EDTA.

Denaturation in 8 M urea and renaturation to active enzyme was also possible for purified Hmd from *M. jannaschii* and *M. kandleri*. After renaturation approximately 40% (*M. jannaschii*) and 90% (*M. kandleri*) of the original activity could be recovered.

3.2. Heterologous production of Hmd in E. coli

Attempts to heterologously express the *hmd* gene from *M. marburgensis* in *E. coli* have failed as revealed by Western blotting [22]. However, it was possible to heterologously express the *hmd* genes from *M. kandleri* and *M. jannaschii*. The

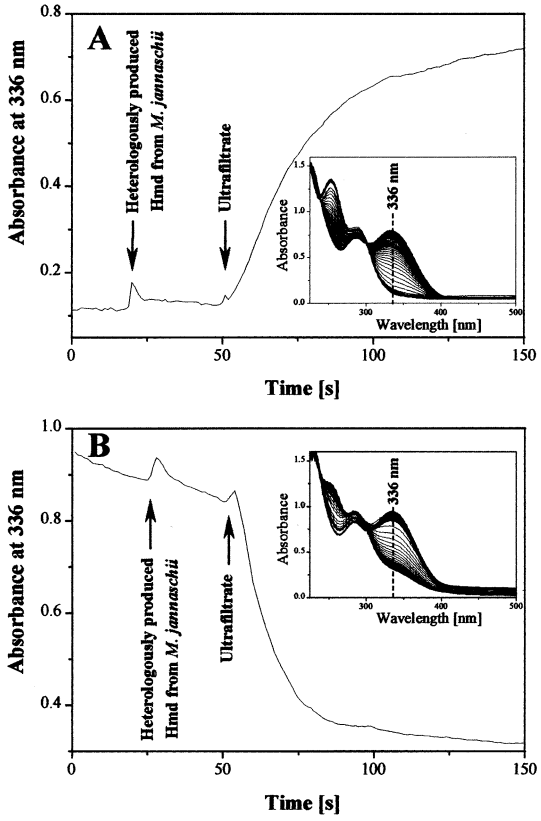

Fig. 2. Enzyme assays showing the activation of heterologously produced Hmd from *M. jannaschii* by ultrafiltrate from active Hmd denatured in 8 M urea. The active Hmd was from *M. marburgensis*. A: Formation of methenyl-H_4MPT^+ from methylene-H_4MPT at pH 6.0 and 65°C followed by measuring the absorbance increase at 336 nm (0.04 μg Hmd; 15 μl ultrafiltrate). B: Formation of methylene-H_4MPT from methenyl-H_4MPT^+ and H_2 at pH 7.5 and 65°C followed by measuring the absorbance decrease at 336 nm (0.2 μg Hmd; 15 μl ultrafiltrate). The slow decrease in absorbance observed prior to the addition of ultrafiltrate was due to the spontaneous hydrolysis of methenyl-H_4MPT^+ under the experimental conditions. The insets show the changes in the UV/visible spectra during the reactions. The spectra were recorded in 1 s time intervals with a Diode-Array detector (Specord S10 from Zeiss) and plotted every 2 s. For detailed assay conditions see Section 2.

heterologously produced enzymes, which were found in the soluble cell fraction, were completely inactive even after unfolding of the purified proteins in urea and subsequent refolding under the conditions described above.

The purified heterologously produced enzyme from *M. kandleri* had an absorbance at 280 nm which was less than 50% of the absorbance shown by the active enzyme purified from *M. kandleri*. It eluted identically from high performance liquid chromatography (HPLC) gel filtration columns as the active enzyme which has a homotetrameric structure [13]. The heterologously produced inactive enzyme and the active enzyme had very similar circular dichroism (CD) spectra indicating similar secondary structures.

3.3. Activation of heterologously produced Hmd by ultrafiltrate

As already indicated, heterologously produced Hmd from *M. jannaschii* and *M. kandleri* was completely inactive. Activity was found, however, when the ultrafiltrate of urea-denatured Hmd, that was purified from *M. marburgensis*, was added to the enzyme assay as outlined in Fig. 1 for Hmd from *M. jannaschii*. In the presence of ultrafiltrate the heterologously produced enzyme catalyzed both the dehydrogenation reaction (Fig. 2A) and the reduction reaction (Fig. 2B). In the absence of Hmd the ultrafiltrate showed no activity. Activity dependence on ultrafiltrate concentration at low enzyme concentration is given in Fig. 3A and activity dependence on enzyme concentration at low ultrafiltrate concentration is given in Fig. 3B. At saturating ultrafiltrate concentration the heterologously produced Hmd from *M. jannaschii* exhibited a specific activity of 1100 U/mg (Fig. 3A). At saturating Hmd concentration (Fig. 3B) the 0.8 ml ultrafiltrate of 0.7 mg denatured Hmd from *M. marburgensis* (1500 U/mg) had the ability to restore 600 U enzyme activity (Fig. 1) or, in other words, the ultrafiltrate exhibited 600 Hmd-activity-restoring-units.

Active ultrafiltrate was also obtained when Hmd from *M. marburgensis* was denatured in 6 M guanidinium chloride containing 20 mM potassium phosphate pH 7.8 and 20 mM β-mercaptoethanol. Ultrafiltrate of cell extract and of heated cell extract from *M. marburgensis* was completely inactive.

The experiment described in Fig. 1 could, with similar results, also be performed with heterologously produced Hmd from *M. kandleri* or with ultrafiltrate of denatured Hmd from *M. jannaschii* or *M. kandleri*. The ultrafiltrate from these enzymes showed, however, only Hmd-activity-restoring-units when the enzymes were denatured in 6 M guanidinium chloride rather than in 8 M urea.

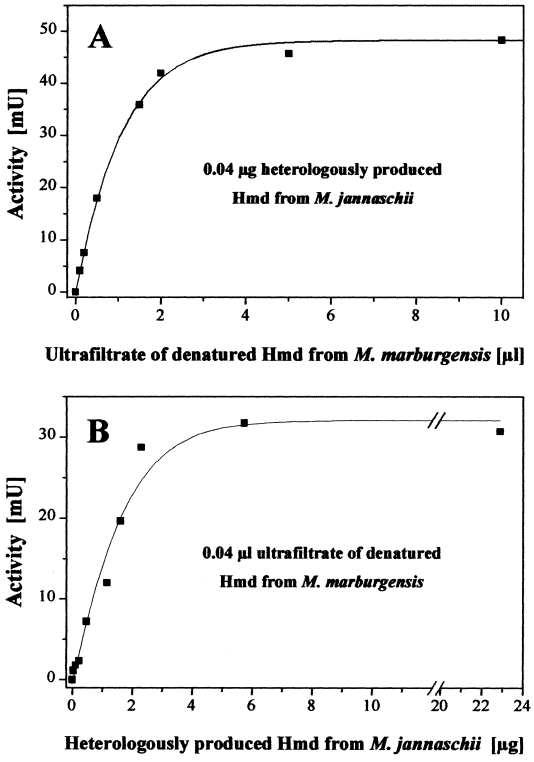

Fig. 3. Activity dependence of heterologously produced Hmd from *M. jannaschii* on the concentration of ultrafiltrate from active Hmd denatured in 8 M urea. The active Hmd was from *M. marburgensis*. A: Plot of the Hmd activity versus the ultrafiltrate concentration at a relatively low Hmd concentration. B: Plot of the activity versus the Hmd concentration at a relatively low ultrafiltrate concentration. Hmd activity was determined in 0.7 ml assay mixtures by measuring the formation of methenyl-H_4MPT^+ from methylene-H_4MPT as described in Fig. 2A. For detailed assay conditions see Section 2.

3.4. Some properties of the activating factor in the ultrafiltrate

The ultrafiltrate that activates heterologously produced inactive Hmd was obtained from urea-denatured active enzyme by using a filter with a 10 kDa molecular mass cut-off. For further investigations the urea was removed by gel filtration as described in Section 2. The recovery of Hmd-activity-restoring-units was approximately 50%. In HPLC analysis of urea-freed ultrafiltrate, the active fractions migrated with an apparent molecular mass well below 1000 Da (Fig. 4). Electrospray ionization mass spectrometry of the urea-freed ultrafiltrate revealed the presence of at least two compounds with molecular masses between 300 and 500 Da (results not shown). The urea-freed ultrafiltrate contained no nickel and only trace amounts of iron and zinc as determined by total reflection X-ray fluorescence spectrometry (TXRF). The activation of Hmd by ultrafiltrate was not inhibited in the presence of EDTA (10 mM) or nitrilotriacetic acid (5 mM) in agreement with the TXRF analysis. No amino acids were found in significant amounts after heating of the urea-freed ultrafiltrate in 6 M HCl for 24 h to 110°C, followed by the addition of fluoren-9-ylmethoxycarbonyl (Fmoc) and separation of the derivatized amino acids via HPLC. The urea-freed ultrafiltrate was thermolabile: at pH 7 approximately 50% of the Hmd-activity-restoring-units were lost within 2 min at 50°C or within 8 min at 30°C. The stability was, however, much higher in the presence of 20 mM β-mercaptoethanol (Fig. 5).

The 0.8 ml ultrafiltrate obtained from 0.7 mg urea-denatured active enzyme showed a UV/visible spectrum with a maximum near 280 nm and an absorbance at 280 nm of 0.4. The absorbance corresponded to that lost by the enzyme upon denaturation and ultrafiltration. The UV absorbing material in the ultrafiltrate eluted from gel filtration columns in several UV absorbing fractions of which only one showed

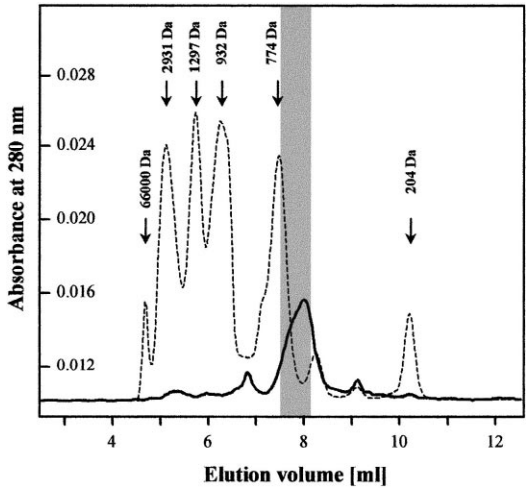

Fig. 4. HPLC gel filtration elution profile of urea-freed ultrafiltrate capable of Hmd activation (solid line). The ultrafiltrate was from denatured Hmd purified from *M. marburgensis*. The fractions, that activated heterologously produced Hmd from *M. jannaschii*, are shaded in gray. The dotted line depicts the elution profile of the molecular mass standards. The 30 cm×7.8 mm G2500 TSK-GEL PW_{XL} column (particle size 6 μm) (TosoHaas) was loaded with 0.15 ml urea-freed ultrafiltrate and eluted at room temperature with 45% acetonitrile in H_2O containing 0.01% trifluoroacetic acid. The flow rate was 0.5 ml/min. The following molecular mass standards were used: bovine serum albumin (66 000 Da); adrenocorticotropic hormone fragment 1–24 (2931 Da); angiotensin I (1297 Da); angiotensin II (932 Da); methenyl-H_4MPT^+ (774 Da); tryptophan (204 Da).

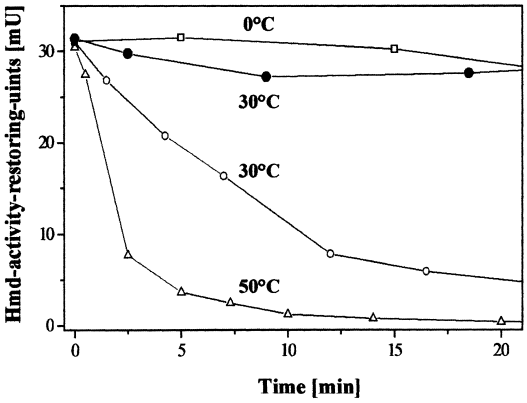

Fig. 5. Kinetics of cofactor inactivation at different temperatures. Urea-freed ultrafiltrate of denatured Hmd from *M. marburgensis* was diluted 1:10 in 1 ml anaerobic 20 mM potassium phosphate pH 7.0 and then incubated at either 0, 30 or 50°C. At the times given 10–50 μl samples were withdrawn and assayed for activity at saturating concentrations of heterologously produced Hmd from *M. jannaschii* (5 μg protein/0.7 ml assay; see Fig. 3B). ●, in the presence of 20 mM β-mercaptoethanol. The activity assays were started by the addition of ultrafiltrate.

Hmd-activity-restoring-units. It is therefore at present not possible to make a statement on the UV-spectroscopic properties of the activating cofactor.

3.5. Conclusion

The results indicate that active Hmd contains a low molecular mass, thermolabile cofactor that is tightly bound to the enzyme but can be released upon enzyme denaturation in urea or guanidinium chloride in the presence of thiol reagents. There were no indications that the cofactor contains a redox-active transition metal. At the moment speculations about the cofactor's nature come too early. We are presently trying to purify the cofactor in sufficient quantities to allow its structure determination. Parallel to that our group is attempting to obtain the crystal structure of active Hmd.

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References

[1] Zirngibl, C., Hedderich, R. and Thauer, R.K. (1990) FEBS Lett. 261, 112–116.
[2] Thauer, R.K., Klein, A.R. and Hartmann, G.C. (1996) Chem. Rev. 96, 3031–3042.
[3] Frey, M. (1998) in: Metal Sites in Proteins and Models, Vol. 90, pp. 97–126, Springer, Berlin.
[4] Fontecilla-Camps, J.C. and Ragsdale, S.W. (1999) in: Advances in Inorganic Chemistry, Vol. 47, pp. 283–333, Academic Press, San Diego, CA.
[5] Thauer, R.K. (1998) Microbiology 144, 2377–2406.
[6] Afting, C., Hochheimer, A. and Thauer, R.K. (1998) Arch. Microbiol. 169, 206–210.
[7] Zirngibl, C., van Dongen, W., Schwörer, B., von Bünau, R.,

Richter, M., Klein, A. and Thauer, R.K. (1992) Eur. J. Biochem. 208, 511–520.
[8] Berkessel, A. and Thauer, R.K. (1995) Angew. Chem. Int. Ed. Engl. 34, 2247–2250.
[9] Geierstanger, B.H., Prasch, T., Griesinger, C., Hartmann, G., Buurman, G. and Thauer, R.K. (1998) Angew. Chem. Int. Ed. Engl. 37, 3300–3303.
[10] Cioslowski, J. and Boche, G. (1997) Angew. Chem. Int. Ed. Engl. 36, 107–109.
[11] Scott, A.P., Golding, B.T. and Radom, L. (1998) New J. Chem. 22, 1171–1173.
[12] Teles, J.H., Brode, S. and Berkessel, A. (1998) J. Am. Chem. Soc. 120, 1345–1346.
[13] Ma, K., Zirngibl, C., Linder, D., Stetter, K.O. and Thauer, R.K. (1991) Arch. Microbiol. 156, 43–48.
[14] Wasserfallen, A., Nolling, J., Pfister, P., Reeve, J. and de Macario, E.C. (2000) Int. J. Syst. Evol. Microbiol. 50, 43–53.
[15] Von Bünau, R., Zirngibl, C., Thauer, R.K. and Klein, A. (1991) Eur. J. Biochem. 202, 1205–1208.
[16] Bult, C.J. et al. (1996) Science 273, 1058–1073.
[17] Breitung, J., Börner, G., Scholz, S., Linder, D., Stetter, K.O. and Thauer, R.K. (1992) Eur. J. Biochem. 210, 971–981.
[18] Escalante-Semerena, J.C., Rinehart Jr., K.L. and Wolfe, R.S. (1984) J. Biol. Chem. 259, 9447–9455.
[19] Afting, C., Kremmer, E., Brucker, C., Hochheimer, A. and Thauer, R.K. (2000) Arch. Microbiol., in press.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: (Sambrook, J., Fritsch, E.F. and Maniatis, T., Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[22] Von Bünau, R. (1994) Ph.D. Thesis, Philipps-Universität, Marburg.