N^5,N^{10}-Methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* has hydrogenase activity

C. Zirngibl, R. Hedderich and R.K. Thauer

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität Marburg, Karl-von-Frisch-Straße, D-3550 Marburg, FRG

Received 12 December 1989

N^5,N^{10}-Methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* (strain Marburg) was purified under anaerobic conditions to apparent homogeneity and a specific activity of approximately 750 μmol/min/mg protein. Polyacrylamide gel electrophoresis under native and denaturing conditions revealed that the enzyme is composed of only one polypeptide with an apparent molecular mass of 43 kDa. The purified enzyme catalyzed the dehydrogenation of N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2=H_4MPT$) (apparent K_m = 20 μM) to N^5,N^{10}-methenyltetrahydromethanopterin ($CH\equiv H_4MPT$) in the absence of any added electron acceptors. One mol of H_2 was generated per mol $CH\equiv H_4MPT$ formed, indicating that protons served as electron acceptor. Coenzyme F_{420}, NAD, NADP and viologen dyes were not reduced by $CH_2=H_4MPT$. The dehydrogenase also catalyzed the reverse reaction, the reduction of $CH\equiv H_4MPT$ to $CH_2=H_4MPT$ with H_2. The data indicate that $CH_2=H_4MPT$ dehydrogenase from *M. thermoautotrophicum* is a novel type of hydrogenase.

Methanopterin; Tetrahydromethanopterin; Methylenetetrahydromethanopterin dehydrogenase; Hydrogenase; Coenzyme F_{420}; *Methanobacterium thermoautotrophicum*

1. INTRODUCTION

Methanobacterium thermoautotrophicum is a thermophilic methanogenic archaebacterium that reduces CO_2 to CH_4. N^5,N^{10}-Methenyltetrahydromethanopterin ($CH\equiv H_4MPT$) and N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2=H_4MPT$) have been shown to be intermediates in this reaction [1]. The reduction of $CH\equiv H_4MPT$ to $CH_2=H_4MPT$ ($E^{o\prime}$ = −390 mV) [2] is catalyzed by $CH_2=H_4MPT$ dehydrogenase (fig.1).

The enzyme has been anaerobically purified by Hartzell et al. [3] and shown to be dependent on coenzyme F_{420}. The specific activity of the preparation obtained was, however, only 0.1 μmol/min/mg. Mukhopadhyay and Daniels [4] and te Brömmelstroet et al. [5] recently described an aerobic purification procedure yielding a coenzyme F_{420}-dependent $CH_2=H_4MPT$ dehydrogenase of much higher specific activity (200 μmol/min/mg). Sodium dodecylsulfate polyacrylamide gel electrophoresis revealed one band at the 32 kDa position [4].

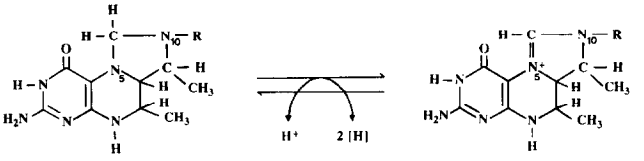
We report here the anaerobic purification of a coenzyme F_{420} independent $CH_2=H_4MPT$ dehydrogenase of apparent molecular mass 43 kDa that catalyzes the reversible reduction of $CH\equiv H_4MPT$ with H_2. A meeting abstract on these findings has been published [6].

Correspondence address: C. Zirngibl, Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität Marburg, Karl-von-Frisch-Straße, D-3550 Marburg, FRG

2. MATERIALS AND METHODS

Tetrahydromethanopterin (H_4MPT) and coenzyme F_{420} were purified from *M. thermoautotrophicum* [7]. $CH_2=H_4MPT$ was generated from H_4MPT and formaldehyde. $CH\equiv H_4MPT$ was prepared by the method of Escalante-Semerena et al. [8]. *M. thermoautotrophicum* (strain Marburg) (DSM 2133) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). The organism was cultivated and harvested and cell extracts were prepared as described [9]. Fast protein liquid chromatography (FPLC) columns and molecular mass standards were from Pharmacia (Freiburg, FRG).

$CH_2=H_4MPT$ dehydrogenase was purified starting from an extract of 25 g cells (wet weight). The purification steps are summarized in table 1. The DEAE Sepharose Fast Flow column (5 × 15 cm) was eluted with an NaCl gradient in 50 mM Tris-HCl, pH 7.6 (in the following referred to as buffer). The dehydrogenase eluted at an NaCl concentration of 0.3 M. The fractions containing activity were pooled, concentrated by ultrafiltration, and diluted with buffer. Portions of 200 mg protein were applied to a Mono Q HR 10/10 column.



$CH_2=H_4MPT$ **$CH\equiv H_4MPT$**

Fig.1. Reaction catalyzed by N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase. $CH_2=H_4MPT$ = N^5,N^{10}-methylenetetrahydromethanopterin; $CH\equiv H_4MPT$ = N^5,N^{10}-methenyltetrahydromethanopterin.

Published by Elsevier Science Publishers B.V. (Biomedical Division)

The dehydrogenase activity eluted at 0.45 M NaCl using a 0.3 M–0.6 M NaCl gradient in buffer. After rechromatography on Mono Q HR 10/10 the enzyme-containing fractions were supplemented with ammonium sulfate to a final concentration of 1 M and applied to a Phenyl Superose HR 10/10 column previously equilibrated with 1 M ammonium sulfate in buffer. The dehydrogenase eluted at 0.3 M ammonium sulfate using a 1–0 M gradient. Final purification was achieved by gel filtration on a Superose 12 column (1.6 × 50 cm) in buffer containing 0.1 M NaCl. The enzyme eluted after 50 ml corresponding to a relative molecular mass of approximately 80000 (± 10000). All purification steps were performed in an anaerobic chamber (Coy, Ann Arbor, MI, USA) containing 95% N_2 and 5% H_2 as gas phase and catalyst for the reductive removal of O_2.

The standard assay for the determination of $CH_2{=}H_4MPT$ dehydrogenation to $CH{\equiv}H_4MPT$ was performed at 60°C in anaerobic cuvettes containing 1 ml of the following reaction mixture: 120 mM potassium phosphate, pH 6; 35 μM H_4MPT; and 3 mM formaldehyde; the gas phase was N_2. After temperature equilibration to 60°C the reaction was started by the injection of enzyme. The formation of $CH{\equiv}H_4MPT$ was followed photometrically at 340 nm (ϵ_{340} = 20800 $cm^{-1} \cdot M^{-1}$) [8]. The standard assay for the determination of $CH{\equiv}H_4MPT$ reduction to $CH_2{=}H_4MPT$ with H_2 was performed at 60°C in anaerobic cuvettes containing 1 ml of the following reaction mixture: 120 ml potassium phosphate, pH 7.5; and 35 μM $CH{\equiv}H_4MPT$; the gas phase was N_2. After temperature equilibration to 60°C enzyme was injected and the reaction was subsequently started by exchange of the gas phase to H_2. The consumption of $CH{\equiv}H_4MPT$ was followed photometrically at 340 nm.

Polyacrylamide gel electrophoreses were performed according to Laemmli [10]. Protein was determined via the Bradford method [11]. H_2 was quantitated after separation by gas chromatography using a thermal conductivity detector [12].

3. RESULTS

3.1. *$CH_2{=}H_4MPT$ dehydrogenase activity in cell extracts*

Cell extracts of *M. thermoautotrophicum* were found to catalyze the dehydrogenation of $CH_2{=}H_4MPT$ to $CH{\equiv}H_4MPT$ in the absence of any added electron acceptors. One mol of H_2 was generated per mol $CH{\equiv}H_4MPT$ formed. A plot of the rates against the protein concentration between 1 μg and 60 μg/ml gave a straight line which passed through zero. This finding indicates that only one independent component in the cell extract was required for the catalysis of $CH_2{=}H_4MPT$ dehydrogenation to $CH{\equiv}H_4MPT$ and H_2. The specific rate of $CH_2{=}H_4MPT$ dehydrogenation was 7 μmol/min/mg protein.

The cell extracts did not catalyze the reduction of coenzyme F_{420}, NAD or NADP with $CH_2{=}H_4MPT$ nor did these coenzymes stimulate $CH{\equiv}H_4MPT$ and H_2 formation from $CH_2{=}H_4MPT$.

M. thermoautotrophicum is known to contain two hydrogenases, a coenzyme F_{420}-reducing enzyme and a methylviologen-reducing enzyme [13–15]. Since $CH_2{=}H_4MPT$ dehydrogenation yielded H_2 the activities of these two enzymes were also determined in cell extract (table 1). The F_{420}-reducing hydrogenase activity was found to be 0.4 μmol/min/mg and the viologen dye-reducing hydrogenase activity was 2.5 μmol/min/mg.

3.2. *Purification of $CH_2{=}H_4MPT$ dehydrogenase*

$CH_2{=}H_4MPT$ dehydrogenase activity was rapidly lost under aerobic conditions. In the presence of dithiothreitol or mercaptoethanol the rate of inactivation by O_2 was considerably enhanced. Therefore, the complete purification procedure was performed under strictly anaerobic conditions in the absence of thiol compounds. The steps employed are summarized in table 1. Via fast protein liquid chromatography (FPLC) a 110-fold purification was achieved yielding an en-

Table 1

Purification of $CH_2{=}H_4MPT$ dehydrogenase from *M. thermoautotrophicum* (strain Marburg) and separation from methylviologen (MV)-reducing hydrogenase and from coenzyme F_{420}-reducing hydrogenase activities

Fraction	Protein (mg)	$CH_2{=}H_4MPT$ dehydrogenase		MV-reducing hydrogenase activity[b] (U)	F_{420}-reducing hydrogenase activity[b] (U)
		Activity (U)	Specific activity (U/mg)		
Cell extract (46 ml)[a]	2500	18000	7	6250	1000
160000 × *g* supernatant	1100	16000	14	5200	900
DEAE Sepharose Fast Flow	528	14000	27	2250	500
Mono Q HR 10/10	125	7000	56	<200	<50
Mono Q HR 10/10 rechromatography	49	5600	115	<1	<1
Phenyl-Superose HR 10/10	10.8	2800	259	<1	<1
Superose 12	2.3	1800	775	<1	<1

1 U = 1 μmol/min

[a] From 25 g cells (wet weight)

[b] Activity in $CH_2{=}H_4MPT$ dehydrogenase fractions

zyme preparation with a specific activity of 775 μmol/min/mg.

The purified enzyme did not catalyze the reduction of coenzyme F_{420} or methylviologen with H_2. The respective hydrogenase activities present in cell extracts were separated from $CH_2{=}H_4MPT$ dehydrogenase during chromatography on DEAE Sepharose Fast Flow (table 1) and Mono Q (fig.2).

After purification, homogeneous enzyme was obtained as judged by polyacrylamide gel electrophoresis under denaturing conditions (fig.3).

The purified enzyme was stored in 50 mM Tris-HCl, pH 7.6, containing 20% glycerol under N_2 as gas phase at 4°C. Under these conditions the enzyme was relatively stable. Only approximately 50% of the activity was lost within a week.

3.3. *Molecular properties of $CH_2{=}H_4MPT$ dehydrogenase*

The molecular mass of the native enzyme was determined by polyacrylamide gradient (5–30%) gel electrophoresis and found to be 45 kDa using aldolase (rabbit muscle) (160 kDa), albumin (bovine serum) (67 kDa), and albumin (egg) (45 kDa) as standards. The protein, which was electro-eluted from the 45 kDa position had $CH_2{=}H_4MPT$ dehydrogenase activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed only one protein band at the 43 kDa position (fig.3). Apparently the $CH_2{=}H_4MPT$ dehydrogenase is composed of only one polypeptide chain.

3.4. *Catalytic properties of $CH_2{=}H_4MPT$ dehydrogenase*

The purified enzyme catalyzed the dehydrogenation of $CH_2{=}H_4MPT$ (apparent K_m = 20 μM) to $CH{\equiv}H_4MPT$ and H_2 (fig.4) and the reverse reaction, the reduction of $CH{\equiv}H_4MPT$ to $CH_2{=}H_4MPT$ with H_2 (fig.5). The time courses of H_2 formation and consumption are given in fig.4A and fig.5A, respectively. $CH{\equiv}H_4MPT$ and $CH_2{=}H_4MPT$ were identified by their characteristic UV/Vis spectra [8] (fig.4B and fig.5B).

The pH optimum for H_2 formation from $CH_2{=}H_4MPT$ was 6.5 (V = 775 μmol/min/mg) and that of $CH{\equiv}H_4MPT$ reduction with H_2 was 7.5 (V = 300 μmol/min/mg). $CH_2{=}H_4MPT$ dehydrogenation was inhibited by its product H_2.

The purified enzyme did not catalyze the reduction of coenzyme F_{420}, NAD, NADP or methylviologen with either $CH_2{=}H_4MPT$ or with H_2.

$CH_2{=}H_4MPT$ dehydrogenase was found to require high ionic strength in the assay for activity, which was essentially zero at very low salt concentrations both in the absence and in the presence of the electron acceptors mentioned above. Upon addition of NaCl or KCl (120 μmol/ml) full activity was immediately restored again.

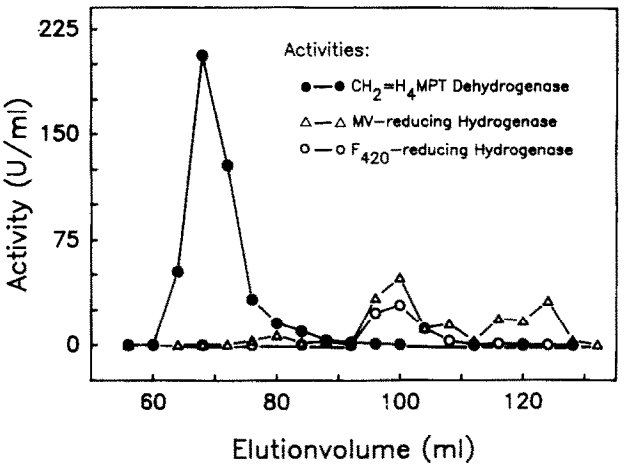


Fig.2. Separation of $CH_2{=}H_4MPT$ dehydrogenase from coenzyme F_{420} reducing hydrogenase and from methylviologen reducing hydrogenase during fast protein liquid chromatography (FPLC) on Mono Q HR 10/10.

3.5. *pH-dependent equilibrium*

The formation of $CH{\equiv}H_4MPT$ and H_2 from $CH_2{=}H_4MPT$ went to completion only at pH values below 6.5. At higher pH the reaction stopped when only part of the substrate had been converted to $CH{\equiv}H_4MPT$ and conversion was essentially zero at pH above 8. When the gas phase was changed from N_2 to H_2 in the assays, the back reaction was observed.

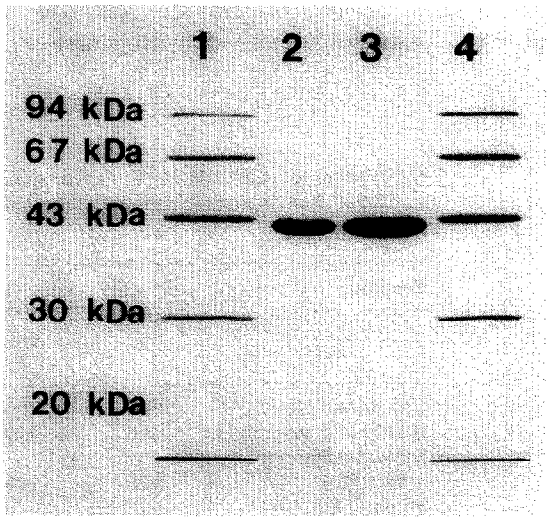


Fig.3. Analysis of purified $CH_2{=}H_4MPT$ dehydrogenase from *M. thermoautotrophicum* by sodium dodecylsulfate polyacrylamide (12%) gel electrophoresis. Lanes 2 and 3, 5 μg and 10 μg purified enzyme, respectively; lanes 1 and 4, molecular mass standards, 3 μg each of phosphorylase b (94 kDa), albumin (bovine serum) (67 kDa), albumin (egg) (43 kDa), carboanhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). The gel was stained with Coomassie Blue R 250.

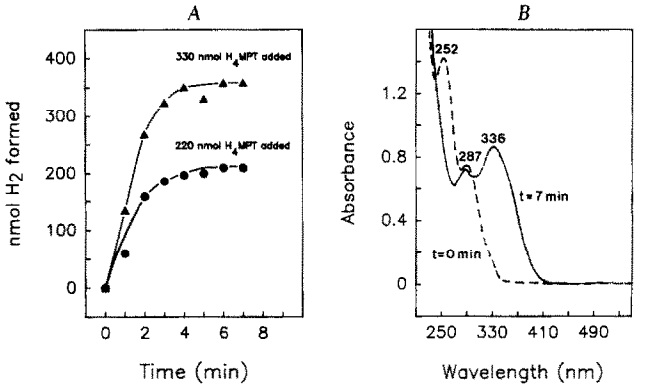


Fig.4. (A) Time course of H_2 formation from $CH_2{=}H_4MPT$ catalyzed by purified $CH_2{=}H_4MPT$ dehydrogenase from *M. thermoautotrophicum*. The 1 ml assays contained: 120 mM potassium phosphate, pH 6; H_4MPT at the concentrations indicated; 3 mM formaldehyde; and 0.5 μg dehydrogenase (230 μmol/min/mg). (B) UV/Vis spectra of the assay solution before start (t = 0 min) and after completion (t = 4 min) of the reaction (samples anaerobically diluted with buffer to a $CH_2{=}H_4MPT$ plus $CH{\equiv}H_4MPT$ concentration of 35 μM).

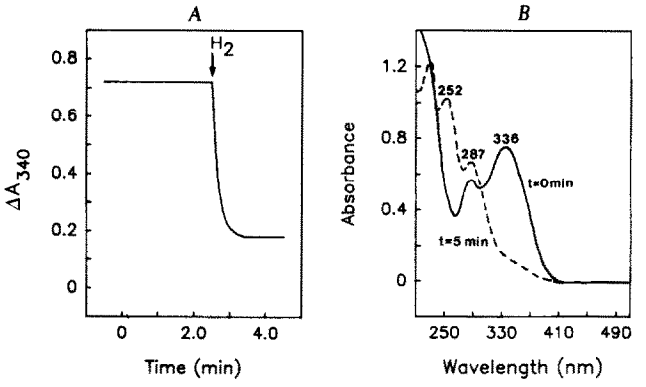


Fig.5. (A) Time course of $CH{\equiv}H_4MPT$ reduction to $CH_2{=}H_4MPT$ with H_2 catalyzed by purified $CH_2{=}H_4MPT$ dehydrogenase from *M. thermoautotrophicum*. The 1 ml assays contained: 120 mM potassium phosphate, pH 7.5; 35 μM $CH{=}H_4MPT$; and 1 μg dehydrogenase (200 μmol/min/mg). (B) UV/Vis spectra of the assay solution before start (t = 0 min) and after completion (t = 5 min) of the reaction.

The reduction of $CH_2{=}H_4MPT$ with H_2 went to completion only at pH values above 7.5. At pH 6.7 and a hydrogen partial pressure of 10^5 Pa equilibrium was attained at a $CH_2{=}H_4MPT$ to $CH{\equiv}H_4MPT$ ratio of approximately 1. These findings indicate that a proton is involved as substrate in $CH_2{=}H_4MPT$ dehydrogenation (see fig.1) and that the free energy change $\Delta G^{\circ\prime}$ of the reaction must be near ± 0 kJ/mol. This is predicted from the standard redox potentials of the $CH{\equiv}H_4MPT/CH_2{=}H_4MPT$ couple ($E^{\circ\prime}$ = −390 mV) [2] and of the H^+/H_2 couple ($E^{\circ\prime}$ = −414 mV).

3.6. *Metal content of $CH_2{=}H_4MPT$ dehydrogenase*

During purification the nickel content of the protein fractions was routinely assayed by atomic absorption spectroscopy (HGA-600 graphite furnace assembly). Nickel was found to co-chromatograph with the F_{420}-reducing hydrogenase, with the methylviologen-reducing hydrogenase and with the methyl-CoM reductase. These three enzymes are known to contain nickel [16]. In the fractions containing $CH_2{=}H_4MPT$ dehydrogenase activity only very little nickel was detected and the small amount present did not correlate with enzyme activity. In the purified enzyme preparation the nickel content was less than 0.1 mol per mol enzyme.

Per mol enzyme approximately 1 mol iron was found. Due to background contamination with iron, which had to be corrected for, there is some uncertainty in this determination. Solutions of the enzyme (0.75 mg/ml in 50 mM Tris-HCl, pH 7.6) were colourless. The UV-spectrum showed a maximum at 280 nm (ΔA = 0.83) with shoulders at 300 nm (ΔA = 0.4) and at 320 nm (ΔA = 0.25), and a minimum at 260 nm (ΔA = 0.7).

4. DISCUSSION

In the results section evidence has been presented that *M. thermoautotrophicum* contains an enzyme catalyzing the following reaction:

$$CH{\equiv}H_4MPT + H_2 \rightleftharpoons CH_2{=}MPT + H^+$$

The enzyme was found to be composed of only one polypeptide chain (fig.3). Because of the substrates used by the protein it can be viewed as being a $CH_2{=}H_4MPT$ dehydrogenase or a $CH{\equiv}H_4MPT$-reducing hydrogenase.

The novel hydrogenase differs from the two other hydrogenases known to be present in *M. thermoautotrophicum* [13–15] and which were separated during the purification procedure (table 1 and fig.2) in that it is unable to mediate the reduction of coenzyme F_{420} or methylviologen with H_2. Thus the enzyme preparation was not contaminated with significant amounts of coenzyme F_{420}-reducing hydrogenase or with methylviologen-reducing hydrogenase. The high specific activity of 775 μmol/min/mg of the purified $CH_2{=}H_4MPT$ dehydrogenase also makes contamination unlikely considering that the specific activity of the $CH_2{=}H_4MPT$ dehydrogenase in cell extracts was significantly higher than that of the two latter hydrogenases (table 1).

It is a general postulate that enzymes mediating the conversion of H_2 contain a transition metal. Nickel-iron and iron-only hydrogenases have been described (for literature see [17,18]). Available evidence indicates that the H_2 forming $CH_2{=}H_4MPT$ dehydrogenase from *M. thermoautotrophicum* contains iron, the exact content of which has, however, not yet been determined. Less than 0.1 mol of nickel per mol enzyme was found.

The observation that cell extracts of *M. thermoautotrophicum* catalyze the dehydrogenation of

$CH_2{=}H_4MPT$ to $CH{\equiv}H_4MPT$ in the absence of added electron acceptors has also been made by Escalante-Semerena et al. [8], by Keltjens et al. [19] and by Mukhopadhyay and Daniels [4]. The electron acceptor used by $CH_2{=}H_4MPT$ dehydrogenase was, however, not determined. Mukhopadhyay and Daniels [4] recently reported that $CH{\equiv}H_4MPT$ formation from $CH_2{=}H_4MPT$ became coenzyme F_{420} dependent when a partially purified enzyme preparation was incubated in the presence of air. Conversion to the F_{420}-dependent form was accelerated in the presence of dithiothreitol or mercaptoethanol. We were able to confirm these observations (unpublished results), the molecular basis of which is presently not understood.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. We thank Marion Enßle for the analyses of the metal content of the protein fractions via atomic absorption spectroscopy.

REFERENCES

[1] Rouvière, P.E. and Wolfe, R.S. (1988) J. Biol. Chem. 263, 7913–7916.
[2] Keltjens, J.T. and Vogels, G.D. (1988) BioFactors 1, 95–103.
[3] Hartzell, P.L., Zvilius, G., Escalante-Semerena, J.C. and Donnelly, M.I. (1985) Biochem. Biophys. Res. Commun. 133, 884–890.
[4] Mukhopadhyay, B. and Daniels, L. (1989) Can. J. Microbiol. 35, 499–507.
[5] Te Brömmelstroet, B.W., Keltjens, J.T., Van der Drift, C. and Vogels, G.D. (1989) Forum Mikrobiol. 12, p.55 (abstract V 121).
[6] Zirngibl, C., Hedderich, R. and Thauer, R.K. (1989) Forum Mikrobiol. 12, p. 88 (abstract P 176).
[7] Börner, G. (1988) Diploma thesis, Marburg.
[8] Escalante-Semerena, J.C., Rinehart, K.L., jr and Wolfe, R.S. (1984) J. Biol. Chem. 259, 9447–9455.
[9] Ellermann, J., Hedderich, R., Böcher, R. and Thauer, R.K. (1988) Eur. J. Biochem. 172, 669–677.
[10] Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Schönheit, P., Moll, J. and Thauer, R.K. (1980) Arch. Microbiol. 127, 59–65.
[13] Graf, E.-G. and Thauer, R.K. (1981) FEBS Lett. 136, 165–169.
[14] Fox, J.A., Livingston, D.J., Orme-Johnson, W.H. and Walsh, C.T. (1987) Biochemistry 26, 4219–4227.
[15] Reeve, J.N., Beckler, G.S., Cram, D.S., Hamilton, P.T., Brown, J.W., Krzycki, J.A., Kolodziej, A.F., Alex, L., Orme-Johnson, W.H. and Walsh, C.T. (1989) Proc. Natl. Acad. Sci. USA 86, 3031–3035.
[16] Thauer, R.K. (1985) Biol. Chem. Hoppe-Seyler 366, 103–112.
[17] Moura, J.J.G., Moura, I., Teixeira, M., Xavier, A.V., Fauque, G.D. and LeGall, J. (1978) in: Metal Ions in Biological Systems (Sigel, H. and Sigel, A. eds) pp.285–314.
[18] Adams, M.W.W., Eccleston, E. and Howard, J.B. (1989) Proc. Natl. Acad. Sci. USA 86, 4932–4936.
[19] Keltjens, J.T., Caerteling, G.C., Van der Drift, C. and Vogels, G.D. (1986) System. Appl. Microbiol. 7, 370–375.