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Ferredoxin-dependent methane formation from acetate in cell extracts of *Methanosarcina barkeri* (strain MS)

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Cell extracts of *Methanosarcina barkeri* grown on acetate catalyzed the conversion of acetyl-CoA to CO_2 and CH_4 at a specific rate of 50 $nmol \cdot min^{-1} \cdot mg^{-1}$. When ferredoxin was removed from the extracts by DEAE-Sephacel anion exchange chromatography, the extracts were inactive but full activity was restored upon addition of purified ferredoxin from *M. barkeri* or from *Clostridium pasteurianum*. The apparent K_m for ferredoxin from *M. barkeri* was determined to be 2.5 μM. A ferredoxin dependence was also found for the formation of CO_2, H_2, and methyl-coenzyme M from acetyl-CoA, when methane formation was inhibited by bromoethanesulfonate. Reduction of methyl-coenzyme M with H_2 did not require ferredoxin. These and other data indicate that ferredoxin is involved as electron carrier in methanogenesis from acetate. Methanogenesis from acetyl-CoA in cell extracts was not dependent on the membrane fraction, which contains the cytochromes.

Methanogenesis from acetate; Acetyl-CoA; Ferredoxin; Coenzyme M; Carbon monoxide dehydrogenase; *Methanosarcina barkeri*

1. INTRODUCTION

M. barkeri is an anaerobic archaebacterium that ferments acetate to CH_4 and CO_2 ($\Delta G^{\circ\prime} = -36$ kJ/mol) [1]. The methyl-group of acetate is converted to CH_4 and the carboxyl-group to CO_2. Acetyl phosphate, acetyl-CoA, methyl-tetrahydromethanopterin (CH_3-H_4MPT), methyl-coenzyme M (CH_3-S-CoM), and a bound carbon monoxide ([CO]) have been identified as intermediates [2–6] (Fig. 1).

Methane formation from the methyl-group of acetyl-CoA is coupled to the oxidation of the carbonyl-group of acetyl-CoA via an electron transport chain (Fig. 1). During electron transport energy is conserved via a chemiosmotic mechanism [7,8]. 7-Mercaptoheptanoyl-threonine phosphate (H-S-HTP) has been identified as one of the electron carriers. H-S-HTP is the immediate electron donor for the reduction of CH_3-S-CoM to CH_4 yielding the heterodisulfide of H-S-CoM and H-S-HTP (CoM-S-S-HTP) as oxidation product [9]. Two other electron carriers believed to participate are cytochromes *b* and *c* [10,11] and ferredoxin [12–14].

The involvement of cytochromes in the electron transport chain is mainly indicated by the finding that only methanogens that can metabolize acetate contain these heme-proteins [11]. The reduction of the cytochromes by CO and their reoxidation by CH_3-S-CoM in cell extracts of *M. barkeri* have been demonstrated [15].

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The involvement of ferredoxin in the electron transport chain is suggested by the finding that all methanogens that can metabolize acetate also contain this iron-sulfur protein.

Ferredoxin has been purified from *M. barkeri* (strain MS) [13], from *M. barkeri* (strain Fusaro) [12] and from *M. thermophila* [14]. Ferredoxin of *M. barkeri* (strain MS) is an iron-sulfur protein composed of 59 amino acids with a calculated molecular mass of 6 kDa of which the sequence has been determined [16]. It shows 41% sequence homology with ferredoxin of *C. pasteurianum* which is a 2 [4Fe-4S] protein. Eight cysteines are involved in cluster formation which are completely conserved in ferredoxin of *M. barkeri*. Despite this fact ferredoxin of *M. barkeri* (strain MS) was found to contain only a [3Fe-3S] cluster [13].

Ferredoxin of *M. barkeri* (strain Fusaro) was calculated to have a molecular mass of 6.1 kDa on the base of the amino acid composition. Indirect evidence is available that this protein contains 2 [4Fe-4S] clusters [12].

Ferredoxin of *M. thermophila* is smaller than the ferredoxin of *M. barkeri*. The molecular mass calculated from the amino acid composition is 4.8 kDa. It was found to contain only 5 cysteines [14]. Terlesky et al. [17] demonstrated that carbon monoxide dehydrogenase of *M. thermophila* uses ferredoxin as electron acceptor.

In this communication evidence is presented that ferredoxin is involved in the electron transport chain connecting the oxidation of the carbonyl-group of acetyl-CoA to CO_2 with the reduction of CH_3-S-CoM to CH_4 in cell extracts of *M. barkeri* (strain MS).

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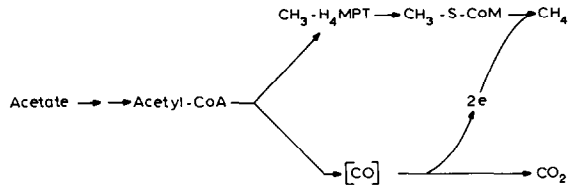

Fig. 1. Pathway of acetate conversion to CH_4 and CO_2 in *M. barkeri*. The oxidation of the carbonyl-group of acetyl-CoA to CO_2 is coupled to the reduction of the methyl-group to CH_4 via an electron transport chain associated with energy conservation. (CH_3-H_4MPT, methyl-tetrahydromethanopterin; CH_3-S-CoM, methyl-coenzyme M; [CO], bound CO).

2. MATERIALS AND METHODS

DEAE-Sephacel (fast flow), Mono Q (HR 10/10) and Superose 12 were from Pharmacia (Freiburg, FRG). Coenzyme M (H-S-CoM = 2-mercaptoethanesulfonate) and metronidazole were from Sigma (Deisenhofen, FRG) and bromoethanesulfonate was from Fluka (Buchs, Switzerland). 5,5′-Dithiobis (2-nitrobenzoid acid) (Ellman's reagent) was from Serva (Heidelberg, FRG). Methyl-coenzyme M, 7-mercaptoheptanoylthreonine phosphate (H-S-HTP) and the heterodisulfide (CoM-S-S-HTP) were synthesized as described [9]. Tetrahydromethanopterin (H_4MPT) was isolated from *Methanobacterium thermoautotrophicum* [18].

M. barkeri (DSM 800) was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). The organism was grown on acetate at 37°C in a 42 liter fermenter (Braun, Melsungen, FRG) containing 30 liter medium [19]. The cells were harvested anaerobically with a continuous flow centrifuge (Heraeus Sepatech Biofuge 17 RS, Osterode, FRG) and cell extracts ($100\,000 \times g$ supernatant) were prepared under strictly anaerobic conditions as described [4,5].

2.1. *Preparation of ferredoxin-free extracts and of ferredoxin*

Cell extract (2 ml; 80 mg protein) was loaded on a DEAE-Sephacel column (1 × 8 cm) previously equilibrated with 50 mM MOPS/KOH buffer pH 7 (10 mM $MgCl_2$; 0.5 mM dithiothreitol). Enzymes were eluted with the same buffer containing 300 mM NaCl. The eluate is designated as ferredoxin-free extract. Ferredoxin was eluted with the same buffer containing 500 mM NaCl. Ferredoxin fractions from about 10 ml cell extract were pooled and further purified by chromatography on Mono Q and Superose 12 [14]. Purified ferredoxin showed a relative molecular mass of 24 kDa determined by gel-filtration on Superose 12. The UV/Visible spectrum at pH 7 of oxidized ferredoxin exhibited maxima at 280 nm and at 390 nm with a shoulder at 312 nm. The ratio of ΔA_{390} to ΔA_{280} was 0.76. For the calculation of the ferredoxin concentration a molar extinction coefficient at 390 nm of 12,800 $M^{-1} \cdot cm^{-1}$ was used [14]. Ferredoxin from *C. pasteurianum* was purified as described ($\epsilon_{390} = 30000\ M^{-1} \cdot cm^{-1}$) [20].

Protein was determined by the method of Bradford [21] using the Bio-Rad microassay [22] with ovalbumin as standard.

2.2. *Assay conditions for ferredoxin-dependent reactions*

All assays were performed in sealed 8 ml serum vials. The reactions were started by addition of cell extract and by transfer from 4°C to 37°C. Incubation was in a shaking water bath at 280 rpm. CH_4, CO_2, and H_2 were quantified after separation by gas chromatography [23].

2.2.1. CH_4- and CO_2-formation from acetyl-CoA

The 0.2 ml assay mixtures contained: 50 mM MOPS/KOH buffer pH 7; 10 mM $MgCl_2$; 30 mM acetyl phosphate; 1 mM coenzyme A; 1 mM coenzyme M; 0.1 mM H_4MPT; 5 mM ATP; 0.3 mM HTP-S-S-HTP (the homodisulfide of H-S-HTP); 0.5 mM dithiothreitol; 0.5 – 2 mg ferredoxin-free extra protein; and ferredoxin as indicated. The gas phase was H_2 at $2 \cdot 10^5$ Pa.

2.2.2. CO_2- and H_2-formation from acetyl-CoA

The 0.2 ml assay mixtures contained: 50 mM MOPS/KOH buffer pH 7; 10 mM $MgCl_2$; 30 mM acetyl phosphate; 1 mM coenzyme A; 10 mM coenzyme M; 0.1 mM H_4MPT; 1 mM bromoethanesulfonate; 0.5 mM dithiothreitol; 1–2 mg ferredoxin-free extract protein; and ferredoxin as indicated. The gas phase was H_2 at $2 \cdot 10^5$ Pa.

2.2.3. CO_2- and H_2-formation from CO

The 0.2 ml assay mixtures contained: 50 mM MOPS/KOH buffer pH 7; 10 mM $MgCl_2$; 1 mM bromoethanesulfonate; 0.5 mM dithiothreitol; 0.5–1 mg ferredoxin-free extract protein; and ferredoxin as indicated. The gas phase was N_2 with 5% CO at $2 \cdot 10^5$ Pa or concentrations indicated in Fig. 3.

2.2.4. CH_4 from CH_3-S-CoM and H_2

The 0.2 ml assay mixtures contained: 50 mM MOPS/KOH buffer pH 7; 10 mM $MgCl_2$, 10 mM CH_3-S-CoM; 0.3 mM HTP-S-S-HTP; 5 mM ATP; 0.5 mM dithiothreitol; and 0.3 mg ferredoxin-free extract; and ferredoxin as indicated. The gas phase was H_2 at $2 \cdot 10^5$ Pa.

2.2.5. CoM-S-S-HTP reduction by CO or by H_2

The 0.2 ml assay mixtures contained: 50 mM MOPS/KOH buffer pH 7; 10 mM $MgCl_2$; 5 mM CoM-S-S-HTP; 1 mM bromoethanesulfonate; 0.01–0.015 ml ferredoxin of *M. barkeri* or of *C. pasteurianum*; and 0.2–0.5 mg ferredoxin-free extract protein. The gas phase was CO or H_2 at $1.5 \cdot 10^5$ Pa. The reaction was followed by analyzing the formation of free SH-groups (H-S-HTP and H-S-CoM) from CoM-S-S-HTP with Ellman's reagent.

Samples (0.025 ml) were withdrawn in 5 min intervals with a syringe and transferred into 0.05 ml 1 M trichloroacetic acid (4°C). After 30 min the precipitated protein was removed by centrifugation. A sample of 0.05 ml of the supernatant was neutralized with 0.05 ml NaOH (0.66 M) and then 0.1 ml Tris/HCl (1 M; pH 8.1), 0.1 ml Ellman's reagent (1 mM; solution in 50 mM sodium acetate, pH 5), and 0.7 ml H_2O were added successively. The absorbance difference against water was measured at 412 nm and corrected for the absorbance difference obtained for the samples withdrawn at $t = 0$. The method was calibrated with coenzyme M and coenzyme A as standards.

2.2.6. Metronidazole-reduction by CO

The assays were performed in sealed 1 ml-cuvettes. The 0.8 ml assay mixtures contained: 50 mM MOPS/KOH buffer pH 7; 10 mM $MgCl_2$; 0.005 mM ferredoxin; 0.1 mM metronidazole; and 0.02 mg ferredoxin-free extract protein. The gas phase was CO at $1.5 \cdot 10^5$ Pa. The reaction was started by the addition of extract protein. The reduction of metronidazole by reduced ferredoxin, which is a spontaneous reaction, was followed at 320 nm ($\epsilon_{320} = 9300\ M^{-1} \cdot cm^{-1}$).

3. RESULTS AND DISCUSSION

3.1. *Ferredoxin-dependent CH_4- and CO_2-formation from acetyl-CoA*

Cell extracts ($100\,000 \times g$ supernatant) of *M. barkeri* catalyzed the formation of CH_4 and CO_2 from acetyl-CoA with specific activities of 40–50 $nmol \cdot min^{-1} \cdot mg^{-1}$. When the extracts were deprived from ferredoxin by adsorption of the iron-sulfur protein to DEAE-Sephacel the activity was less than 1 $nmol \cdot min^{-1} \cdot mg^{-1}$. Upon re-addition of ferredoxin, full activity was restored. The apparent K_m for ferredoxin was determined to be 2.5 μM (Fig. 2, Table I). Ferredoxin isolated from *C. pasteurianum* could substitute for ferredoxin from *M. barkeri* (app. $K_m = 0.5$ μM). Viologen dyes could not replace ferredoxin; they even proved to be in-

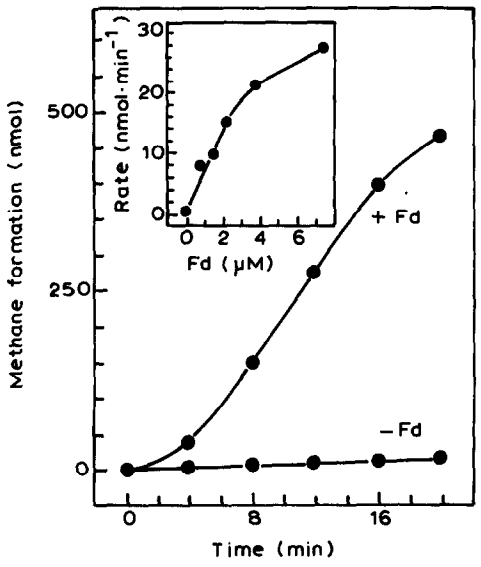

Fig. 2. Methane formation from acetyl-CoA catalyzed by ferredoxin-free extracts of *M. barkeri*. Time course in the absence and presence of ferredoxin (Fd) from *M. barkeri*. The inset shows the rate dependence on the ferredoxin concentration. The assays contained 0.58 mg protein of ferredoxin-free extract and 7.5 μM ferredoxin or the ferredoxin concentrations indicated. For assay conditions see Materials and Methods.

hibitory. Also, Ti(III)citrate (25 mM) [6] could not substitute for ferredoxin. The results indicate that methanogenesis from acetyl-CoA in cell extract is dependent on ferredoxin.

(a) $CH_3CO\text{-}S\text{-}CoA + H_2O \xrightarrow{Fd} CH_4 + CO_2 + H\text{-}S\text{-}CoA$
$\Delta G^{\circ\prime} = -71.7$ kJ/mol [24]

Methanogenesis from acetyl-CoA in cell extracts of *M. barkeri* was dependent on the presence of H_2 [2]. An explanation for the H_2 requirement was recently provided by the finding that in cell extracts the reaction is composed of two partial reactions which are electrically coupled via molecular hydrogen [6].

(b) $CH_3CO\text{-}S\text{-}CoA + H\text{-}S\text{-}CoM + H_2O \longrightarrow CH_3\text{-}S\text{-}CoM + CO_2 + H\text{-}S\text{-}CoA + H_2$
$\Delta G^{\circ\prime} = +13.3$ kJ/mol [24]

(c) $CH_3\text{-}S\text{-}CoM + H_2 \longrightarrow CH_4 + H\text{-}S\text{-}CoM$
$\Delta G^{\circ\prime} = -85.0$ kJ/mol [24]

We therefore determined which of the two partial reactions was dependent on ferredoxin.

3.2. *Ferredoxin-dependent CO_2- and H_2-formation from acetyl-CoA under conditions of inhibited CH_4-formation*

CH_4-formation from acetyl-CoA was inhibited by bromoethanesulfonate, which inhibits CH_3-S-CoM reduction to CH_4 [1]. Under these conditions CO_2 and H_2 were formed in stoichiometric amounts and CH_3-S-CoM accumulated [6,25]. The specific activity in cell extracts of *M. barkeri* was 20–30 nmol · min^{-1} · mg^{-1}. In ferredoxin-free extracts the activity was less than 1 nmol · min^{-1} · mg^{-1} but full activity could be restored upon addition of ferredoxin (Table I). Methylviologen (0.05 mM) rather than Ti(III) citrate (25 mM) [6] could substitute for ferredoxin. These results indicate that ferredoxin is required for CO_2- and H_2-production in partial reaction b.

Indirect evidence is available that partial reaction b is catalyzed by carbon monoxide dehydrogenase [26–28] which also is involved in the conversion of CO plus H_2O to CO_2 plus H_2. Cell extracts catalyzed the latter reaction at a specific rate of 100–150 nmol · min^{-1} · mg^{-1}. Ferredoxin-free extracts were only active after supplementation with the iron-sulfur-protein (Table I). For ferredoxin of *M. barkeri* and ferredoxin of *C. pasteurianum* apparent K_m values of 10 μM and 2.5 μM, respectively, were determined. Thus H_2- and CO_2-formation from CO plus H_2O showed the same ferredoxin-dependence as CO_2- and H_2-formation from acetyl-CoA.

Cell extracts of *M. barkeri* catalyzed the reduction of ferredoxin by CO at a specific rate of 1–2 μmol ·

Table I

Ferredoxin-dependent and ferredoxin-independent reactions catalyzed by cell extracts of *M. barkeri*

Reaction	Ferredoxin dependent	app. K_m (μM)	Specific rate (nmol/min · mg)
CH_4 and CO_2 from acetyl-CoA	+	2.5	40–50
CO_2 and H_2 from acetyl-CoA	+	2.5	20–30
CO_2 and H_2 from CO	+	10.0	100–150
CH_4 from CH_3-S-CoM and H_2	–	–	100–120
CoM-S-S-HTP reduction by H_2	–	–	50–80
CoM-S-S-HTP reduction by CO	+	n.d.	200–300
Metronidazole reduction by CO	+	n.d.	1000–2000

The experiments were performed with ferredoxin-free extracts supplemented with ferredoxin of *M. barkeri*. For assay conditions see Materials and Methods
n.d. = not determined

$min^{-1} \cdot mg^{-1}$ as deduced from the rate of ferredoxin-dependent metronidazole reduction by CO. Purified carbon monoxide dehydrogenase from *M. barkeri* catalyzed the reaction at a specific rate of 40–60 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (unpublished results). These and the above findings suggest that ferredoxin is the direct electron acceptor in the oxidation of the carbonyl group of acetyl-CoA to CO_2.

The rate of H_2- and CO_2-formation from CO was highest at a CO concentration in the gas phase of 5% (Fig. 3). Higher CO concentrations inhibited the reaction probably by negatively affecting the hydrogenase activity, which is known to be inhibited by CO [29].

3.3. *Ferredoxin-independent reduction of CH_3-S-CoM to CH_4 by H_2*

Ferredoxin-free cell extract was found to catalyze the reduction of CH_3-S-CoM to CH_4 with H_2 at a specific rate of 80–100 $nmol \cdot min^{-1} \cdot mg^{-1}$. Upon addition of ferredoxin the specific rate increased by only 10–20%. Ferredoxin could be substituted by Ti(III)citrate (5 mM) [6] in this stimulatory effect. These findings indicate that ferredoxin does not directly participate in partial reaction c.

It has recently been shown that the reduction of CH_3-S-CoM with H_2 (reaction c) proceeds in two steps: the reduction of CH_3-S-CoM with 7-mercaptoheptanoylthreonine phosphate (H-S-HTP) to methane and CoM-S-S-HTP [9] (reaction d); and the reduction of the heterodisulfide with H_2 [30] (reaction e).

(d) $CH_3S\text{-}CoM + H\text{-}S\text{-}HTP \longrightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}HTP$
$\Delta G^{\circ\prime} = -45$ kJ/mol [24]

(e) $CoM\text{-}S\text{-}S\text{-}HTP + H_2 \longrightarrow H\text{-}S\text{-}CoM + H\text{-}S\text{-}HTP$
$\Delta G^{\circ\prime} = -40$ kJ/mol [24]

Both reactions were catalyzed by ferredoxin-free extracts, the activities (approximately 100 $nmol \cdot min^{-1} \cdot mg^{-1}$) being only slightly stimulated by the addition of ferredoxin as to be expected from the finding that CH_3-S-CoM reduction with H_2 was ferredoxin-independent.

When CO rather than H_2 was used as electron donor, the reduction of CoM-S-S-HTP required ferredoxin (Table I) in agreement with the property of carbon monoxide dehydrogenase to use ferredoxin as electron acceptor (see above). CoM-S-S-HTP reduction by CO proceeded at a specific rate of 200–300 $nmol \cdot min^{-1} \cdot mg^{-1}$. The dependence of the rate on the CO concentration is given in Fig. 3.

3.4. *On the role of cytochromes*

All the experiments described above were performed in cell extracts which had been centrifuged for one hour at $100\,000 \times g$ to remove the membrane fraction, in which the cytochromes are located [10,11,15]. The membrane fraction was not required for activity: With

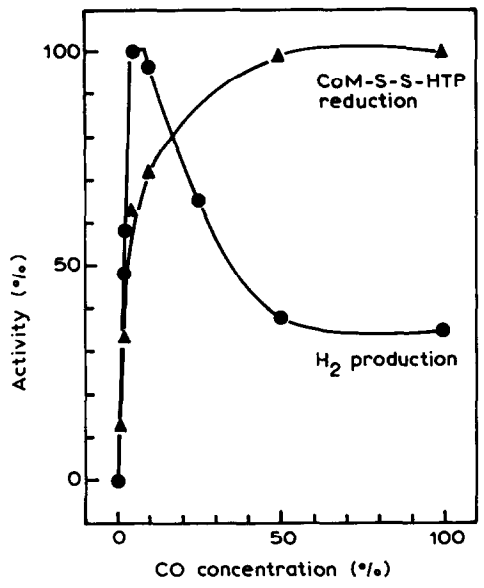

Fig. 3. H_2 production from CO and CoM-S-S-HTP reduction by CO in cell extracts of *M. barkeri*. The assays contained 15 μM ferredoxin of *C. pasteurianum*. 100% activity was 90 $nmol \cdot min^{-1}$ (0.8 mg cell extract protein) for H_2 production from CO at 5% CO in the gas phase and 110 $nmol \cdot min^{-1}$ (0.4 mg cell extract protein) for CoM-S-S-HTP reduction by CO at 100% CO in the gas phase.

cell extract centrifuged at $27\,000 \times g$ essentially the same results as with the $100\,000 \times g$ supernatant were obtained. Also the activities were not enhanced when the membrane fraction was re-added to the $100\,000 \times g$ supernatant. These findings indicate that probably cytochromes were not required for methane formation from acetate in cell extracts of *M. barkeri*.

Methane formation from acetate in intact cells is coupled with energy conservation [8], is sodium ion dependent [31], is inhibited by sodium ionophores [8], and is inhibited by H_2 [6,27]. The in vivo reaction thus differs considerably from the in vitro reaction which is not coupled with energy conservation, which is not affected by sodium ionophores [5], and which requires H_2 to proceed [2,6]. The finding that cytochromes are not involved in methanogenesis from acetyl-CoA in cell extracts thus does not exclude a function of the heme-iron proteins in methanogenesis from acetate in intact cells.

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REFERENCES

[1] Rouvière, P.E. and Wolfe, R.S. (1988) J. Biol. Chem. 263, 7913–7916.
[2] Krzycki, J.A. and Zeikus, J.G. (1984) FEMS Microbiol. Letters 25, 27–32.

[3] Nelson, M.J.K. and Ferry, J.G. (1984) J. Bacteriol. 160, 526–532.
[4] Fischer, R. and Thauer, R.K. (1988) FEBS Letters 228, 249–253.
[5] Fischer, R. and Thauer, R.K. (1989) Arch. Microbiol. 151, 459–465.
[6] Fischer, R. and Thauer, R.K. (1990) Arch. Microbiol. 153, 156–162.
[7] Blaut, M. and Gottschalk, G. (1985) Trends in Biochem. Sci. 10, 486–489.
[8] Peinemann, S., Müller, V., Blaut, M. and Gottschalk, G. (1988) J. Bacteriol. 170, 1369–1372.
[9] Ellermann, J., Hedderich, R., Böcher, R. and Thauer, R.K. (1988) Eur. J. Biochem. 172, 669–677.
[10] Kühn, W. and Gottschalk, G. (1983) Eur. J. Biochem. 135, 89–94.
[11] Kühn, W., Fiebig, K., Hippe, H., Mah, R.A., Huser, B.A. and Gottschalk G. (1983) FEMS Microbiol. Lett. 20, 407–410.
[12] Hatchikian, E.C., Bruschi, M., Forget, N. and Scandellari, M. (1982) Biochem. Biophys. Res. Commun. 109, 1316–1323.
[13] Moura, I., Moura, J.J.G., Huynh, B., Santos, H., LeGall, J. and Xavier, V. (1982) Eur. J. Biochem. 126, 95–98.
[14] Terlesky, K.C. and Ferry, J.G. (1988) J. Biol. Chem. 263, 4080–4082.
[15] Kemner, J.M., Krzycki, J.A., Prince, R.C. and Zeikus, J.G. (1987) FEMS Microbiol. Lett. 48, 267–272.
[16] Hausinger, R.P., Moura, I., Moura, J.J.G., Xavier, A.V., Santos, M.H., LeGall, J. and Howard, J.B. (1982) J. Biol. Chem. 257, 14192–14197.
[17] Terlesky, K.C., Ferry, J.G. (1988) J. Biol. Chem. 263, 4075–4079.
[18] Börner, G. (1988) Diploma thesis, Marburg.
[19] Karrasch, M., Bott, M. and Thauer, R.K. (1989) Arch. Microbiol. 151, 137–142.
[20] Schönheit, P., Wäscher, C. and Thauer, R.K. (1978) FEBS Lett. 89, 219–222.
[21] Bradford, M.M. (1976) Anal. Biochem. 76, 248–254.
[22] Bio-Rad Laboratories (1981) Instruction manual.
[23] Schönheit, P., Moll, J. and Thauer, R.K. (1980) Arch. Microbiol. 127, 59–65.
[24] Thauer, R.K. (1990) Biochim. Biophys. Acta, in press.
[25] Grahame, D.A. and Stadtman, T.C. (1987) Biochem. Biophys. Res. Commun. 147, 254–258.
[26] Krzycki, J.A., Lehman, L.J. and Zeikus, J.G. (1985) J. Bacteriol. 163, 1000–1006.
[27] Eikmanns, B. and Thauer, R.K. (1984) Arch. Microbiol 138, 365–370.
[28] Terlesky, K.C., Barber, M.J., Aceti, D.J. and Ferry, J.G. (1987) J. Biol. Chem. 262, 15392–15395.
[29] Adams, M.W.W., Mortenson, L.E. and Chen, J.S. (1981) Biochim. Biophys. Acta 594, 105–176.
[30] Hedderich, R. and Thauer, R.K. (1988) FEBS Lett. 234, 223–227.
[31] Perski, H.J., Schönheit, P. and Thauer, R.K. (1982) FEBS Lett. 143, 323–326.