Biochemistry of methanogenesis: a tribute to Marjory Stephenson

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Historical overview

In 1933, Stephenson & Stickland (1933a) published that they had isolated from river mud, by the single cell technique, a methanogenic organism capable of growth in an inorganic medium with formate as the sole carbon source.

\[
4\text{HC}O_2^- + 4\text{H}^+ \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O} \quad \Delta G^\circ = -1445 \text{kJ mol}^{-1}
\]

Methane formation from formate was shown to occur in a stepwise manner, by the preliminary decomposition of formic acid into CO₂ and H₂, followed by a reduction of CO₂ by H₂, suggesting that formate was not an intermediate in the reduction of CO₂ to methane.

\[
\text{HCOO}^- + \text{H}^+ \rightarrow \text{H}_2 + \text{CO}_2 \quad \Delta G^\circ = -35 \text{kJ mol}^{-1}
\]

\[
4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \quad \Delta G^\circ = -131 \text{kJ mol}^{-1}
\]

Cell suspensions of the micro-organism catalysed the reduction of methylene blue with H₂, indicating that the methanogen contained an enzyme which activates molecular hydrogen.

\[
\text{H}_2 \rightarrow 2\text{e}^- + 2\text{H}^+ \quad F_0' = -414 \text{mV}
\]

This enzyme had been discovered by Stephenson & Stickland (1931a) 2 years before in a number of bacterial species and was named by them 'hydrogenase'.

The paper by Stephenson & Stickland (1933a) is considered to mark the beginning of the modern era for study of methanogenesis (Wolfe, 1993). It is the first report on the isolation in pure culture of a methanogen and the first study of an enzyme and of reactions involved in methanogenesis.

Besides CO₂, the cell suspensions of the methanogen isolated by Stephenson & Stickland (1933a) catalysed the reduction of carbon monoxide, formaldehyde (added as hexamethylenetetramine) and methanol to methane with H₂, albeit at much lower rates than the reduction of CO₂. It is significant that the cell suspensions did not yield methane from compounds of two or more carbon atoms. The cell suspensions did, however, slowly reduce sulphate with H₂ to H₂S, a property not encountered by any other methanogen isolated since then. It therefore seems possible that, in spite of all efforts to purify it, the culture obtained by Stephenson & Stickland (1933a) was contaminated with one of the sulphate reducers abundantly present in the river mud (Stephenson & Stickland, 1931b) from which the methanogen was isolated (Elsden & Pirie, 1949).

The following review summarizes what we presently know about the biochemistry of methanogenesis, emphasis being put on the enzyme methyl-coenzyme M reductase catalysing the methane-forming reaction proper. It is a tribute to Marjory Stephenson, who discovered hydrogenase in these micro-organisms. Marjory Stephenson died 50 years ago on 12 December 1948 at the age of only 63 (Elsden & Pirie, 1949; Robertson, 1949; Woods, 1950; Elsden, 1981). She had worked for 30 years in the Cambridge Biochemical Laboratory headed by Frederick Gowland Hopkins (Stephenson, 1948) on various aspects of the chemistry of bacteria (Stephenson, 1932, 1933, 1934, 1935). The 3rd edition of her book on *Bacterial Metabolism* (Stephenson, 1949) is a treasury of knowledge very worth reading even today, 50 years later. Marjory Stephenson was the second woman to be elected to the Royal Society of London (Mason, 1992, 1996) and a cofounder of the Society for General Microbiology, of which she was the second president (Postgate, 1995).
In this review, with the exception of a few papers describing discoveries, only publications on the biochemistry of methanogenesis are cited that have appeared since 1993: these contain reference to earlier and often more important papers, many of which have come from the laboratories of A. J. Kluyver (Kamp et al., 1959), H. A. Barker (Barker, 1956), T. Stadtman (Stadtman, 1967; Grahame & Stadtman, 1993), R. Wolfe (Wolfe, 1991, 1996), J. D. Ferry (Ferry, 1993b, 1995, 1997a), G. Gottschalk (Deppenmeier et al., 1996), A. Klein (Sorgenfrei et al., 1997a), J. Reeve (Reeve et al., 1997) and G. Vogels (Keltjens & Vogels, 1993). A historical overview of methanogenesis has recently been published by Wolfe (1993). The reader is also referred to the book on Methanogenesis: Ecology, Physiology, Biochemistry & Genetics edited by Ferry (1993a), to the review by Daniels (1993) and by Blaut (1994) and three more recent reviews by the author (Weiss & Thauer, 1993; Thauer et al., 1993; Thauer, 1997).

**Methanogens: where they live, what they do and who they are**

Methane is an end product of the microbial decomposition of organic matter in anaerobic freshwater environments such as lake sediments and the intestinal tract of animals. Estimates are that presently about 1% of the plant material formed per year by photosynthesis from CO₂ is remineralized via methane, more than 10⁹ tons of the combustible gas being intermediately generated. Roughly two-thirds of the methane diffuses into aerobic zones, where it is oxidized by methanotrophic bacteria. A few per cent is buried, leading to the formation of methane deposits, but most of the rest escapes to the atmosphere where it is photochemically converted to CO₂. The concentration of atmospheric methane has increased steadily during the last 300 years from <0.7 to <1.5 p.p.m. above normal levels, owing probably in large part to the expanded cultivation of rice and ruminant livestock (Conrad, 1996). This is of concern since methane is a potent greenhouse gas.

In anaerobic freshwater sediments, plant material such as glucose from cellulose is completely decomposed to CO₂ and CH₄.

\[
\text{Glucose} \rightarrow 3\text{CO}_2 + 3\text{CH}_4 \quad \Delta G^\circ = -418.1 \text{ kJ mol}^{-1}
\]

This reaction is not catalysed by single micro-organisms but by syntrophic associations of micro-organisms. First the glucose is fermented to acetate, CO₂ and H₂ or to acetate, formate and H₂:

\[
\text{Glucose} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{H}^- + 2\text{CO}_2 + 4\text{H}_2 \quad \Delta G^\circ = -2157 \text{ kJ mol}^{-1}
\]

\[
\text{Glucose} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{H}_2\text{COO}^- + 4\text{H}^- + 2\text{H}_2 \quad \Delta G^\circ = -2087 \text{ kJ mol}^{-1}
\]

These fermentations are brought about by strictly anaerobic bacteria and/or protozoa. In a second step, the products of glucose fermentation are then converted to methane, the rate of conversion being such that the concentrations of acetate (<1 mM), formate (<0.1 mM) and H₂ (<1 μM) in the anaerobic sediments remain very low (Zinder, 1993).

\[
\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{CH}_4 \quad \Delta G^\circ = -36 \text{ kJ mol}^{-1}
\]

\[
4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \quad \Delta G^\circ = -131 \text{ kJ mol}^{-1}
\]

\[
4\text{HCOO}^- + 4\text{H}^+ \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O} \quad \Delta G^\circ = -144.5 \text{ kJ mol}^{-1}
\]

The organisms mediating these methane-forming reactions were originally thought to be bacteria, but are now recognized as belonging to a separate phylogenetic domain, the **Archaea**. Methanoarchaea are highly specialized. They can only use acetate, H₂ and CO₂, and/or other C₁ compounds such as methanol, methylthiols and methylamines as energy substrates, the C₁ compounds being either disproportionated to CO₂ and CH₄ or reduced to methane with H₂ as exemplified for methanol:

\[
4\text{CH}_3\text{OH} + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + 1\text{CO}_2 + 4\text{H}_2\text{O} \quad \Delta G^\circ = -106.5 \text{ kJ (mol CH}_4^-1
\]

\[
\text{CH}_3\text{OH} + \text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O} \quad \Delta G^\circ = -112.5 \text{ kJ mol}^{-1}
\]

Methanogenesis is the only way that methanogenic archaea can obtain energy for growth and these are the only organisms known to produce methane as a catalytic end product.

Despite the high specialization, not all methanogens are phylogenetically closely related. This is reflected, for example, in differences in cell wall composition (Sprott & Beveridge, 1993; Kandler & König, 1998). Methanogens can also have very different optimal growth conditions; thus some thrive optimally at temperatures below 20 °C and others at or near the temperature of boiling water (Boone et al., 1993).

Methanogens taxonomically all belong to the archael kingdom of **Euryarchaeota**. They are classified in five orders each phylogenetically related to another as distantly as the **Cyanobacteria** to the **Proteobacteria**. The five orders are: **Methanobacterales**, **Methanococcales**, **Methanomicrobiales**, **Methanopyrales** and **Methanosarcinales**. Of these, only the **Methanosarcinales** can ferment acetate to CO₂ and CH₄ and grow on methanol, methylthiols or methylamines as sole energy source. In turn, hyperthermophilic species are only found among the **Methanobacterales**, **Methanococcales** and **Methanopyrales** (Boone et al., 1993).

The DNA sequence of the total genome has, until now, been determined for two methanoarchaea, for **Methanococcus jannaschii** (Bult et al., 1996) and for **Methanobacterium thermoautotrophicum** (strain ΔH) (Smith et al., 1997). Recently, the sequence for **Archaeoglobus fulgidus** has also been published (Klenk et al., 1997). This sulphate-reducing archaean is phylogenetically most closely related to the **Methanosarcinales** with which it has many biochemical features in common.
Table 1. Reactions and enzymes known to be involved in methane formation from acetate in the *Methanosarcinales* (Ferry, 1993b, 1995, 1997a)

<table>
<thead>
<tr>
<th>Reaction†</th>
<th>Enzyme (gene)</th>
<th>Most recent literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate + CoA → acetyl-CoA + H₂O</td>
<td>Acetate kinase (ack) and phosphotransacetylase (pta) in <em>Methanosarcina</em> spp. or acetate thiol kinase (acs) in <em>Methanosaeta</em> spp.</td>
<td>Latimer &amp; Ferry (1993); Singh-Wissmann &amp; Ferry (1995); Eggen et al. (1991a)</td>
</tr>
<tr>
<td>Acetyl-CoA + H₂SPT → CH₃H₂SPT + CO₂ + CoA + 2[H]</td>
<td>Carbon-monoxide dehydrogenase/acetyl-CoA synthase</td>
<td>Grahame (1993); Sowers et al. (1993); Lu et al. (1994); Grahame &amp; DeMoll (1995, 1996); Eggen et al. (1991b, 1996); Grahame et al. (1996); Maupin-Furlow &amp; Ferry (1996a, b)</td>
</tr>
<tr>
<td>CH₃H₂SPT + H₂S-CoM → CH₃S-CoM + H₂SPT</td>
<td>Methyl-H₂SPT:coenzyme M methyltransferase (energy conserving)</td>
<td>Fischer et al. (1992); Lu et al. (1995); Lienard et al. (1996); Lienard &amp; Gottschalk (1998)</td>
</tr>
<tr>
<td>CH₃S-CoM + H₂S-CoB → CoM-S-CoB + CH₄</td>
<td>Methyl-coenzyme M reductase††</td>
<td>Thauer et al. (1993); Springer et al. (1995)</td>
</tr>
<tr>
<td>CoM-S-CoB + 2[H] → H₂S-CoM + H₂S-CoB</td>
<td>Heterodisulphide reductase†† (hdrDE)</td>
<td>Heiden et al. (1993); Peer et al. (1994); Künkel et al. (1998)</td>
</tr>
</tbody>
</table>

† ΔG₀ values add up to -38 kJ mol⁻¹, which is very similar to the ΔG₀ = -36 kJ mol⁻¹ calculated for CH₃COO⁻ + H⁺ → CH₄ + CO₂ from free energy of formation data (Thauer et al., 1977). See also the first footnotes to Tables 2 and 3.

‡ From Thauer et al. (1977).

§ From Thauer (1990); CO₂ in the gaseous state at 10⁵ Pa. E'* was determined by Grahame & DeMoll (1995) to be +200 mV and ΔG₀ to be +28 kJ mol⁻¹, values consistent with the overall thermodynamics of methanogenesis from acetate (see first footnote), from CO₂ (Table 3) and from methanol (Table 3). At first sight, a ΔG₀ = +41.3 kJ mol⁻¹ appears unrealistically endergonic. Note, however, that in the reaction four products are formed from two substrates. ΔG₀ is thus highly concentration dependent. At 0.1 mM concentrations of all substrates and products (pH₅ = 10 Pa), ΔG₀ = -26 kJ mol⁻¹ and E' = -340 mV (Thauer, 1990).

¶ The nickel-iron-sulphur corrinoid enzyme is isolated from *Methanosarcina* spp. as a multienzyme complex composed of five different subunits (Grahame & DeMoll, 1996; Maupin-Furlow & Ferry, 1996a, b); ferredoxin appears to be the physiological electron acceptor (Fischer & Thauer, 1990; Peer et al., 1994; Ferry, 1995, 1997a). The encoding genes in *Methanosarcina thermophila* are organized in a transcription unit, *cdhABCXDE*, X being an open reading frame of unknown function (Maupin-Furlow & Ferry, 1996b). CdhA and CdhC are predicted to be nickel iron-sulphur proteins and CdhD and CdhE to harbour the corrinoid prosthetic group (Künkel et al., 1998).

‖ From Weiss et al. (1994).


** From Thauer et al. (1993).

†† The enzyme is isolated as a multi-enzyme complex of three different subunits, McrABG, in an αβγδ configuration.

†† Membrane-associated enzyme purified from methanol-grown *Methanosarcina Barkeri* is a complex of two different subunits, HddRE (Künkel et al., 1997), and from acetate-grown cells is a complex of eight different subunits, HddDE and EchABCDEF, Ech being an *E. coli* hydrogenase-3-type hydrogenase (Künkel et al., 1998). HddRE is a b-type cytochrome (Künkel et al., 1997, 1998).

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(Schwörer et al., 1993a; Kunow et al., 1993, 1994, 1995; Thauer & Kunow, 1995; Vorholt et al., 1995, 1997a).

Noteworthy is a recent hypothesis for the origin of eukaryotic cells from strictly hydrogen-dependent autotrophic methanoarchaea (Martin & Müller, 1998; see also Vogel, 1998).

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**Energy metabolism of methanoarchaea**

The reactions and enzymes known to be involved in methane formation from acetate, from CO₂ and from methanol are summarized in Tables 1–3.

The energy metabolism of methanogens can be viewed
**Table 2. Reactions and enzymes known to be involved in methane formation from CO₂ (Thauer et al., 1993)**

MFR, methanofuran; H-S-CoM, coenzyme M; H-S-CoB, coenzyme B; H₄MPT, tetrahydromethanopterin, which is a tetrahydrofolate (H₄F) analogue found instead of H₄F in the *Methanobacteriales* (for structures see Fig. 3). In the other orders, modified forms of H₄MPT are present: tetrahydroscarcinapterin in the *Methanosarcinales* and *Methanococcales* and tattiopterin and/or thermopterin in the *Methanomicrobicales* (Gorris & van der Drift, 1994). F₄20H₂ is a 5' deazaflavin derivative found in relatively high concentrations in methanogenic archaea (Gorris & van der Drift, 1994). See also the legend to Table 4.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme (gene)</th>
<th>Most recent literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ + MFR + 2[H] → formyl-MFR</td>
<td>Formylmethanofuran</td>
<td>Schmitz et al. (1994); Bertram &amp; Thauer (1994); Bertram et al. (1994a, b); Wasserfallen (1994); Hochheimer et al. (1995, 1996); Vorholt et al. (1996, 1997b); Vorholt &amp; Thauer (1997); Holm &amp; Sander (1997)</td>
</tr>
<tr>
<td>Methenyl-H₄MPT⁺ + F₄20H₂ → methylene-H₄MPT + F₄20H₂</td>
<td>F₄20H₂-dependent methylene-H₄MPT dehydrogenase (mtd)</td>
<td>Klein et al. (1993b); Kunow et al. (1993); Klein &amp; Thauer (1995, 1997); Mukhopadhyay et al. (1995)</td>
</tr>
<tr>
<td>Methenyl-H₄MPT⁺ + H₂ → methylene-H₄MPT + H₂</td>
<td>H₂-forming methylene-H₄MPT dehydrogenase (hmd)</td>
<td>Schwörer et al. (1993b); Schleucher et al. (1994, 1995); Klein et al. (1995a, b); Berkessel &amp; Thauer (1995); Hartmann et al. (1996a, b); Thauer et al. (1996)</td>
</tr>
<tr>
<td>Methylene-H₄MPT + F₄20H₂ → CH₃⁺H₂MPT + F₄20H₂</td>
<td>F₄20-dependent methylene-H₄MPT reductase (mer)</td>
<td>Vaapel &amp; Thauer (1995); Nolling et al. (1995a); Kunow et al. (1993)</td>
</tr>
<tr>
<td>CH₃⁺H₂MPT + F₄20H₂ → CH₃S-CoM + CH₄</td>
<td>Methyl-H₄MPT: coenzyme M methyl-transferase (energy conserving)</td>
<td>Stupperich et al. (1993); Gärtner et al. (1993, 1994); Weiss et al. (1994); Harms et al. (1995); Harms &amp; Thauer (1996a, 1997, 1998)</td>
</tr>
<tr>
<td>CH₃S-CoM + H-S-CoM → CH₃-S-CoM + H₂MPT</td>
<td>Methyl-coenzyme M reductase</td>
<td>Bonacker et al. (1993); Goubeaud et al. (1997); Shima et al. (1997); Ermler et al. (1997b); Becker &amp; Ragsdale (1998)</td>
</tr>
<tr>
<td>CH₃S-CoM + H-S-CoB → CoM-S-CoM + CH₄</td>
<td>Heterodisulphide reductase (hdrA, hdrBC)</td>
<td>Hedderich et al. (1994); Setzke et al. (1994)</td>
</tr>
</tbody>
</table>

*ΔG⁰⁺ values from Thauer et al. (1993). They add up to −130.7 kJ mol⁻¹, which is almost identical to the ΔG⁰⁻ = −131 kJ mol⁻¹ calculated for 4H₂ + CO₂ → CH₄ + 2H₂O from free energy of formation data (Thauer et al., 1977).*

† From Bertram & Thauer (1994); determined at 60 °C; CO₂ in the gaseous state at 10⁶ Pa.

‡ Calculated for 2[H] = 1H₂ at 10⁶ Pa. The direct electron donor is probably a polyferredoxin (Vorholt et al., 1996). The endergonic reduction of CO₂ with H₂ to formylmethanofuran is driven by the electrochemical Na⁺ potential involving reversed electron transport (Kaiser & Schönheit, 1989a, b).

§ *fud* denotes the genes encoding the tungsten enzyme and *fmd* the molybdenum enzyme in *Methanobacterium thermoautotrophicum* (Hochheimer et al., 1996). The latter enzymes are isolated from *Methanobacterium thermoautotrophicum* as multienzyme complexes composed of the subunits FwdABC and FwdA+FmdBC, respectively. FwdB and FmdB contain bound molybdopterin-guanine dinucleotide and tungsten and molybdenum, respectively (Hochheimer et al., 1996).

|| H₂ + F₄20H₂ → F₄20H₂⁺; ΔG⁰⁺ = −11 kJ mol⁻¹; the Eₚ of the F₄20H₂/F₄20H₂⁺ couple is −360 mV (Thauer et al., 1993) and that of the H²+/H₂ couple is −414 mV (Thauer et al., 1977).
to consist of two parts (Fig. 1): an oxidative part in which coenzyme M (H-S-CoM, 2-thioethanesulphonate) and coenzyme B (H-S-CoB, 7-thioheptanoylthreonine-phosphate) (for structures see Fig. 4) are oxidized to the heterodisulphide CoM-S-S-CoB; and a reductive part in which the heterodisulphide of coenzyme M and coenzyme B is re-reduced.

**Oxidative part**

In this part, methyl-coenzyme M is a central intermediate (Taylor & Wolfe, 1974a; b; Shapiro & Wolfe, 1980; Lovley et al., 1984). It is formed from coenzyme M and acetate, CO$_2$ or reduced C$_1$ compounds such as methanol, methylthiols and methylamines via the pathways shown in Fig. 2. Methyl-coenzyme M is subsequently reduced with coenzyme B to methane with the concomitant formation of the heterodisulphide of coenzyme M and coenzyme B (Bobik et al., 1987; Ellermann et al., 1988).

\[ \text{CH}_3\text{-S-CoM + H-S-CoB} \rightarrow \text{CH}_4 + \text{CoM-S-S-CoB} \]

\[ \Delta G^\circ = -45 \text{ kJ mol}^{-1} \]

As far as known the exergonic reaction, which is catalysed by methyl-coenzyme M reductase and which proceeds essentially irreversibly, is not coupled with energy conservation (Thauer et al., 1993).

In methanogenesis from CO$_2$ (Escalante-Semerena et al., 1984) and from acetate (Fischer & Thauer, 1989), N$^5$-methyltetrahydromethanopterin (CH$_3$-H$_4$MPT) or N$^5$-methyltetrahydroarcinopterin (CH$_3$-H$_4$SPT) is an intermediate in methyl-coenzyme M formation (Fig. 2). [Tetrahydromethanopterin (H$_4$MPT) is the tetrahydrofolate (H$_4$F) analogue found instead of H$_4$F in the *Methanobacteriales* (for structures see Fig. 3). In the other orders, modified forms of H$_4$MPT are present: tetrahydroarcinopterin in the *Methanosarcinales* and *Methanococcales* and tatioprotein and/or thiotoprotein in the *Methanomicrobiales* (Gorris & van der Drift, 1994; see also White, 1998).] The methyl group of CH$_3$-H$_4$MPT is transferred to coenzyme M in an exergonic reaction (\[ \Delta G^\circ = -30 \text{ kJ mol}^{-1} \]) coupled with energy conservation via an electrochemical sodium potential (Gartner et al., 1994; Peinemann et al., 1990; Deppenmeier et al., 1990a, b, 1991; Sparling et al., 1993). Dependent on the methanogenic growth substrate, the electrons for the reduction derive from the oxidation of either H$_2$, the carboxyl group of acetyl-CoA, formate, ethanol or 2-propanol or of one of the reduced C$_1$ compounds methanol, methylthiols or methylamines. Under standard conditions, the free energy change associated with heterodisulphide reduction with H$_2$ is \[-40 \text{ kJ mol}^{-1}, \]

with formate is \[-43.5 \text{ kJ mol}^{-1}, \]

with ethanol (acetate as product) is \[-35 \text{ kJ mol}^{-1} \]

and with methanol (CO$_2$ as product) is \[-34 \text{ kJ mol}^{-1} \]

which is sufficient to drive the phosphorylation of 1 mol ADP (\[ \Delta G^\circ = +31.8 \text{ kJ mol}^{-1} \]) (Thauer et al., 1977). The reduction of the heterodisulphide with acetyl-CoA (\[ \Delta G^\circ = +0 \text{ kJ mol}^{-1} \]) (calculated from $E_0^\circ$ values given in Table 1) and with 2-propanol (\[ \Delta G^\circ = -15 \text{ kJ mol}^{-1} \]) is much less exergonic. Under the conditions in the cell, the free energy change $AG$ is, however, probably considerably more negative since the reduction is “pushed” by irreversible formation of the heterodisulphide from methyl-coenzyme M and coenzyme B. Such a coupling is not unusual: e.g. in the arginine dehydroase pathway operative in many bacteria and archaea, the energy-conserving formation of ornithine, CO$_2$, and NH$_3$ from citrulline (\[ \Delta G^\circ = -19 \text{ kJ mol}^{-1} \]) is also “pushed” by the preceding reaction, the irreversible hydrolysis of arginine to citrulline and NH$_3$ (\[ \Delta G^\circ = -37.7 \text{ kJ mol}^{-1} \]) (Thauer et al., 1977). There is evidence that heterodisulphide reduction with H$_2$ is coupled with the synthesis of more than 1 mol ATP (Deppenmeier et al., 1996).

**Reductive part**

Since the generation of the heterodisulphide in the oxidative part of the energy metabolism apparently is not coupled with net ATP formation, the energy required for growth of methanogens must be generated in the reductive part, the exergonic reduction of the heterodisulphide (Fig. 1), and indeed it has been shown that the reduction of the heterodisulphide is coupled with phosphorylation of ADP via the chemiosmotic mechanism involving an electrochemical H$^+$ potential as intermediate (Peinemann et al., 1990; Deppenmeier et al., 1990a, b, 1991; Sparling et al., 1993). Dependent on the methanogenic growth substrate, the electrons for the reduction derive from the oxidation of either H$_2$, the carboxyl group of acetyl-CoA, formate, ethanol or 2-propanol or of one of the reduced C$_1$ compounds methanol, methylthiols or methylamines. Under standard conditions, the free energy change associated with heterodisulphide reduction with H$_2$ is \[-40 \text{ kJ mol}^{-1}, \]

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*From Weiss et al. (1994).*

*Membrane-associated multienzyme complex composed of eight different subunits, MtrA–H, MtrA harbouring a corrinoid prosthetic group (Harms et al., 1995). Energy conservation proceeds via an electrochemical Na$^+$ potential (Gärtner et al., 1994). For literature on the enzyme complex from *Methanosarcina* spp., see Table 1.*

*mcr* denotes the genes encoding the isoenzyme I and mrt the isoenzyme II. Both isoenzymes are isolated as multienzyme complexes composed of three different subunits, McrABG or MrtABG, in an $\alpha_2\beta_2\gamma_2$ configuration. For literature on the enzyme complex from *Methanosarcina* spp., see Table 1.

*Calculated for [H]$^+$ = H$_2$ at 10$^5$ Pa.*

*The enzyme is purified from *Methanobacterium thermoautotrophicum* in a complex with the F$_{100}$ non-reducing hydrogenase (Setzke et al., 1994).*
Heterodisulphide reduction with H₂

The reduction of the heterodisulphide with the primary electron donors involves at least two enzymes, a dehydrogenase and a heterodisulphide reductase, and an electron transport chain connecting the two enzymes as schematically exemplified for heterodisulphide reduction with H₂:

\[
\text{H₂} \rightarrow 2\text{e}^- + 2\text{H}^+ \\
2\text{e}^- + 2\text{H}^+ + \text{CoM-S-S-CoB} \rightarrow \text{H-S-CoM} + \text{H-S-CoB} \\
E^\circ = -200 \text{ mV}
\]
Fig. 1. Energy metabolism of methanogenic archaea. In the oxidative part, coenzyme M (H-S-CoM) and coenzyme B (H-S-CoB) are oxidized to the heterodisulphide CoM-S-S-CoB by CO₂, acetate or reduced C₁ compounds (CH₃-X) such as methanol, methylthiols and methylamines, which in turn are reduced to CH₄. In the reductive part, the heterodisulphide is reduced to coenzyme M and coenzyme B, the electron transport from the electron donors being coupled with phosphorylation.

Fig. 2. Pathways of methyl-coenzyme M (CH₃-S-CoM) formation from acetate, CO₂ and reduced C₁ compounds (CH₃-X) such as methanol, methylthiols and methylamines. H-S-CoM, coenzyme M; [CO], enzyme-bound CO; CH₃-H₄MPT, N⁵-methyltetrahydromethanopterin. Tetrahydromethanopterin (H₄MPT) is a tetrahydrofolic acid (H₄F) analogue found instead of H₄F in the Methanobacteriales (for structures see Fig. 3). In the other orders, modified forms of H₄MPT are present: tetrahydrosarcinapterin in the Methanosarcinales and Methanococcales and tatiopterin or thermopterin in the Methanomicrobiales (Gorris & van der Drift, 1994).

The heterodisulphide reductase (Hdr), first discovered by Hedderich & Thauer (1988), is a novel type of disulphide reductase catalysing the reduction of the heterodisulphide in two successive one-electron steps and probably involving thyl radical intermediates (Künkel et al., 1997; Heim et al., 1998). The enzyme complex, composed of at least two different subunits, has been characterized from Methanobacterium thermoautotrophicum (Hedderich et al., 1990, 1994; Setzke et al., 1994), from Methanosarcina Barkeri (Heiden et al., 1993, 1994; Künkel et al., 1997, 1998) and from Methanosarcina thermophila (Peer et al., 1994). Heterodisulphide reductase HdrDE from Methanosarcina spp. is a cytochrome b (HdrE)-containing enzyme (Heiden et al., 1993, 1994; Künkel et al., 1997); methanophenazine is probably the direct electron donor, the oxidation of reduced methanophenazine with heterodisulphide being coupled with energy conservation (Abken et al., 1998a, b; Bäumer et al., 1998). The enzyme HdrABC from Methanobacterium spp. is an iron–sulphur protein (Hedderich et al., 1994; Setzke et al., 1994). The direct electron donor is not yet known (see Nolling et al., 1995c).

Fig. 3. Structures of tetrahydromethanopterin (H₄MPT) (Gorris & van der Drift, 1994; Schleucher et al., 1994) and of tetrahydrofolic acid (H₄F). The functionally most important difference between H₄MPT and H₄F is that H₄MPT has an electron-donating methylene group conjugated to N' via the aromatic ring whereas H₄F has an electron-withdrawing carbonyl group in this position. As a consequence, the redox potential E₉₀= -390 mV of the N⁵,N¹⁰-methenyl-H₄MPT/N⁵,N¹⁰-methylene-H₄MPT couple is by 90 mV more negative than the E₉₀= -300 mV of the N⁵,N¹⁰-methenyl-H₄F/N⁵,N¹⁰-methylene-H₄F couple and the redox potential E₉₀= -320 mV of the N⁵,N¹⁰-methyl-H₄MPT/N⁵,N¹⁰-methylene-H₄MPT couple is by 120 mV more negative than the E₉₀= -200 mV of the N⁵,N¹⁰-methyl-H₄F/N⁵,N¹⁰-methylene-H₄F couple (Thauer et al., 1996). Tetrahydrosarcinapterin (H₄SPT) is a H₄MPT derivative with a glutamyl group attached to the hydroxyglutaryl group in the side chain.

Hydrogenotrophic methanogens can contain up to four different types of hydrogenases and of each type, several isoenzymes (Thauer et al., 1993; Sorgenfrei et al., 1997a, b; Künkel et al., 1998; for topology see Braks et al., 1994): (i) F₄₃0-reducing Ni/Fe-hydrogenase (Vaupel & Thauer, 1998); (ii) F₄₃0 non-reducing hydrogenase of unknown physiological electron acceptor – in Methanosarcina spp., one of the subunits from the enzyme is a cytochrome b (Deppenmeier, 1995; Deppenmeier et al., 1995; Kumazawa et al., 1994; Kenner & Zeikus, 1994a, b) and in Methanobacterium spp., which are devoid of cytochromes, the electron acceptor is possibly either a 44 kDa polyferrodoxin (Reeve et al., 1989;
Table 4. Dehydrogenases catalysing the reduction of F<sub>420</sub> and the oxidation of F<sub>420H<sub>2</sub></sub> in the energy metabolism of methanogenic archaea

F<sub>420</sub> is a 5' deazaflavin derivative found in relatively high concentration in methanogenic archaea (Gorris & van der Drift, 1994). F<sub>420</sub> in methanogens is converted to inactive F<sub>420H</sub> by adenylation and F<sub>420H</sub> to F<sub>420</sub> by deadenylation in response to different growth conditions (Vermeij et al., 1994, 1995, 1996).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
<th>Most recent literature</th>
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<tr>
<td>1. Reduction of F&lt;sub&gt;420&lt;/sub&gt;</td>
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<tr>
<td>F&lt;sub&gt;420&lt;/sub&gt;-reducing hydrogenase</td>
<td>All hydrogenotrophic methanogens</td>
<td>Sorgenfrei et al. (1997a, b); Vaupel &amp; Thauer (1998)</td>
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<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent formate dehydrogenase</td>
<td>Methanogens growing on formate</td>
<td>Nölling &amp; Reeve (1997); Grahame &amp; Stadtman (1993)</td>
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<tr>
<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent methylene-H&lt;sub&gt;4&lt;/sub&gt;MPT dehydrogenase</td>
<td>Methanogens growing on methanol, methylthiols or methyamines</td>
<td>Keltjens &amp; Vogels (1993)</td>
</tr>
<tr>
<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent methylene-H&lt;sub&gt;4&lt;/sub&gt;MPT reductase</td>
<td>Methanogens growing on methanol, methylthiols or methylamines</td>
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<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;-forming methylene-H&lt;sub&gt;4&lt;/sub&gt;MPT dehydrogenase plus F&lt;sub&gt;420&lt;/sub&gt;-dependent methylene-H&lt;sub&gt;4&lt;/sub&gt;MPT dehydrogenase</td>
<td>Most hydrogenotrophic methanogens</td>
<td>Thauer et al. (1996); Afting et al. (1998)</td>
</tr>
<tr>
<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent alcohol dehydrogenase</td>
<td>Methanogenium liminatans, Methanococcales and Methanopyrales</td>
<td>Klein et al. (1996); Widdel &amp; Frimmer (1995); Berk et al. (1996)</td>
</tr>
<tr>
<td>NADP-dependent alcohol dehydrogenase plus F&lt;sub&gt;420&lt;/sub&gt;-dependent NADP reductase</td>
<td>Methanococcus palustre, Methanocorpusculum spp.</td>
<td>Berk et al. (1997); Widdel &amp; Frimmer (1995)</td>
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<tr>
<td>2. Oxidation of F&lt;sub&gt;420H&lt;sub&gt;2&lt;/sub&gt;&lt;/sub&gt;</td>
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<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent methylene-H&lt;sub&gt;4&lt;/sub&gt;MPT dehydrogenase</td>
<td>Methanogens reducing CO&lt;sub&gt;2&lt;/sub&gt; to methane</td>
<td>See Table 2</td>
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<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent methylene-H&lt;sub&gt;4&lt;/sub&gt;MPT reductase</td>
<td>Methanogens reducing CO&lt;sub&gt;2&lt;/sub&gt; to methane</td>
<td>See Table 2</td>
</tr>
<tr>
<td>F&lt;sub&gt;420H&lt;sub&gt;2&lt;/sub&gt;&lt;/sub&gt; dehydrogenase complex</td>
<td>Methanosaicina mazei, Methanolobus tindarius</td>
<td>Haase et al. (1992); Abken &amp; Deppenmeier (1997); Abken et al. (1998a, b)</td>
</tr>
<tr>
<td>F&lt;sub&gt;420&lt;/sub&gt;-dependent hydrogenase</td>
<td>Methanococcales and Methanobacteriales growing on formate and lacking F&lt;sub&gt;420H&lt;/sub&gt;&lt;sub&gt;2&lt;/sub&gt; dehydrogenase complex</td>
<td>Vaupel (1993)</td>
</tr>
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Hedderich et al., 1992; Nölling et al., 1995c) or a 45 kDa flavoprotein (Wasserfallen et al., 1995; Nölling et al., 1995c); (iii) Escherichia coli hydrogenase-3-type Ni/Fe-hydrogenase of unknown physiological electron acceptor (Kinkel et al., 1998); and (iv) a metal-free hydrogenase, the H<sub>2</sub>-forming methylenetetrahydro-methanopterin dehydrogenase, which together with the F<sub>420</sub>-dependent methylenetetrahydromethanopterin dehydrogenase catalyses the reduction of F<sub>420</sub> with H<sub>2</sub> (Thauer et al., 1996; Reeve et al., 1997). Both enzymes are induced during growth under conditions of nickel deprivation (Afting et al., 1998).

There are indications that at least two of the hydrogenases can be involved in heterodisulphide reduction with H<sub>2</sub>: with inside-out vesicles from Methanosarcina mazei it has been shown that both F<sub>420</sub>-dependent (Deppenmeier et al., 1990a, b) and F<sub>420</sub>-independent (Deppenmeier et al., 1991) reduction of the heterodisulphide with H<sub>2</sub> are coupled with the phosphorylation of ADP (Deppenmeier et al., 1996; Abken et al., 1998a, b).

Heterodisulphide reduction with F<sub>420H<sub>2</sub></sub>

Cell extracts of Methanosarcina mazei and of Methanolobus tindarius catalyse the reduction of heterodisulphide with reduced F<sub>420</sub>, the coenzyme of many dehydrogenases in methanogenic archaea (Table 4). (F<sub>420</sub> is a 5' deazaflavin derivative with an 360 mV present in relatively high concentrations in these organisms; Gorris & van der Drift, 1994.) For the reduction of the heterodisulphide with F<sub>420H</sub><sub>2</sub>, besides heterodisulphide reductase an additional enzyme is required: Methanosarcina spp. (Deppenmeier et al., 1990a, b; Abken & Deppenmeier, 1997) and Methanolobus tindarius (Deppenmeier et al., 1990b; Haase et al., 1992) have been shown to contain a membrane-associated F<sub>420H<sub>2</sub></sub> dehydrogenase complex catalysing...
the reduction of methanophenazine with $F_{440}H_2$, the reduction being coupled with phosphorylation of ADP (Abken et al., 1998a, b; Bäumer et al., 1998). Such an $F_{440}H_2$ dehydrogenase complex is also found in Archaeoglobus sp. (Kunow et al., 1993, 1994; Klenk et al., 1997), but is apparently lacking in Methanococcus spp. (Bult et al., 1996; and Methanobacterium sp. (Smith et al., 1997) as deduced from the genome sequences. In methanogens lacking the $F_{440}H_2$ dehydrogenase complex and not growing on $H_2$ and CO$_2$, heterodisulphide reduction with $F_{440}H_2$ probably proceeds via $H_2$ as intermediate (Vaupe1, 1993).

**Coupling with ATP synthesis**

As indicated, methyl transfer from $N^2$-methyltetrahydromethanopterin to coenzyme M in the oxidative part of the energy metabolism is coupled with the build-up of an electrochemical Na$^+$ gradient (Becher et al., 1992a, b; Lienard et al., 1996). In turn, the reduction of the heterodisulphide in the reductive part is coupled with electrogenic $H^+$ translocation (Peinemann et al., 1990; Deppeynmeier et al., 1990a, b, 1991; Sparling et al., 1993). Methanosarcina spp. have been shown to contain an $H^+$-translocating $A_1A_0$ ATPase (Wilms et al., 1996; Inatomi, 1996; Ruppert et al., 1998) and there is indirect evidence also for the presence of an $Na^+$-translocating $F_0F_1$ ATPase (Becher & Müller, 1994). Methanobacterium spp. and Methanococcus spp. harbour only one $A_1A_0$ ATPase as deduced from the genome sequences (Bult et al., 1996; Smith et al., 1997). It is not known whether this ATPase is a $Na^+$ - or $H^+$-translocating. There is evidence for both (Chen & Konisky, 1993; Sparling et al., 1993; Smigan et al., 1994). Methanogens contain an $Na^+/H^+$ antiporter capable of interconversion of the electrochemical $H^+$ and $Na^+$ potentials (Schönheit & Beimborn, 1983; Deppeynmeier et al., 1996).

The stoichiometry of coupling of methanogenesis with ADP phosphorylation appears not to be constant. Evidence is available that during growth of methanogens on $H_2$ and CO$_2$ the growth yield [$g$ (mol CH$_4$,mol$^{-1}$)] increases with decreasing $H_2$ concentrations (Schönheit et al., 1980; Morgan et al., 1997; for regulation see Pihl et al., 1994; Nölling et al., 1995a, b; Nölling & Reeve, 1997), indicating that at low $H_2$ concentrations coupling of heterodisulphide reduction with ADP phosphorylation is tighter than at high $H_2$ concentrations. With decreasing $H_2$ concentrations the free energy change associated with CO$_2$ reduction to methane decreases from $\sim 131$ kJ mol$^{-1}$ at 10$^4$ Pa $H_2$ to only approximately $\sim 30$ kJ mol$^{-1}$ at the $H_2$ concentrations prevailing in methanogenic ecosystems where the $H_2$ partial pressure is only between 1 and 10 Pa.

**Other heterodisulphide-generating reactions**

Methyl-coenzyme M reduction with coenzyme B is not the only reaction in which the heterodisulphide is regenerated. Most methanogens contain a fumarate reductase which catalyses the reduction of fumarate with H-S-CoM and H-S-CoB to succinate and CoM-S-S-CoB (Bobik & Wolfe, 1989; Heim et al., 1998). This reaction is involved in the biosynthesis of 2-oxoglutarate, which, in the methanogens containing the fumarate reductase, is synthesized via reductive carboxylation of succinyl-CoA (Tersteegen et al., 1997; Thompson et al., 1998).

**Analogy between heterodisulphide reduction and sulphur reduction**

Many anaerobic bacteria and archaea can grow with elemental sulphur as terminal electron acceptor in the energy metabolism (Schauder & Kröger, 1993). Sulphur reduction to $H_2S$ is coupled with energy conservation as evidenced by growth on $H_2$ and $S^0$. In elemental sulphur, which at room temperature is $S_8$, the sulphur atoms are connected via S-S bonds just as the two sulphurs in heterodisulphide. The redox potential of the $S^0/H_2S$ couple and that of the heterodisulphide/H-S-CoM+H-S-CoB couple are both in the order of $\sim -200$ mV (Thauer et al., 1977). Methanogens and sulphur-reducing micro-organisms thus have in common that they both live at the expense of S-S bond reduction. Methanogens differ, however, from sulphur reducers in that they are independent of an external sulphur source as electron acceptor since they can reoxidize the 'reduced sulphur' with CO$_2$ or one of the other methanogenic carbon substrates, which themselves are concomitantly reduced to methane.

**Methyl-coenzyme M, an intermediate unique to methanogenesis**

Coenzyme M and methyl-coenzyme M have, until now, only been found in methanogenic archaea (Balch & Wolfe, 1979). The same holds true for the enzymes catalysing the formation of methyl-coenzyme M (Table 5) and for methyl-coenzyme M reductase. All the other enzymes and coenzymes involved in methanogenesis, maybe with exception of coenzyme B, are also present in sulphate-reducing archaea (Vorholt et al., 1995, 1997a; Klenk et al., 1997). Recently, tetrahydromethanopterin and tetrahydromethanopterin-dependent enzymes were even found in a methylotrophic z-proteobacterium (Chistoserdova et al., 1998), indicating that some of the methanogenic enzymes and coenzymes are not as unique to the archaeal kingdom as previously thought. Also of interest is the recent finding that several non-methanogens, including *Bacillus subtilis*, contain genes encoding a putative heterodisulphide reductase, the function of which remains to be elucidated (Heim et al., 1998).

Coenzyme M is the smallest coenzyme known to date. The enzymes catalysing its methylation and reductive demethylation are highly specific for its structure. 2-Selenoethanesulphonate and 3-thiopropionate can in part substitute for H-S-CoM in its function, 3-thiopropanesulphonate cannot (Gunsalus et al., 1978; Wackett et al., 1987; Tallant & Krzycki, 1996, 1997).
For abbreviations, see Table 2.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme system</th>
<th>Most recent literature</th>
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<tbody>
<tr>
<td>( \text{CH}_3\text{H}_2\text{MPT} + \text{H-S-CoM} \rightarrow \text{CH}_3\text{S-CoM} + \text{H}_2\text{MPT} )</td>
<td>N(^5)-Methyl-( \text{H}_2\text{MPT} : \text{coenzyme M} ) methyltransferase (energy conserving) (MtrA–H)</td>
<td>Stupperich et al. (1993); Gärtner et al. (1993, 1994); Weiss et al. (1994); Harms et al. (1995); Harms &amp; Thauer (1996a, 1997, 1998); Lu et al. (1995); Lienard et al. (1996); Lienard &amp; Gottschalk (1998)</td>
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<td>( \Delta G^\circ = -30 \text{kJ mol}^{-1} )</td>
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<td>( \text{CH}_2\text{OH} + \text{H-S-CoM} \rightarrow \text{CH}_2\text{S-CoM} + \text{H}_2\text{O} )</td>
<td>Methanol : coenzyme M methyltransferase (MtaA + MtaBC)†</td>
<td>Sauer et al. (1997); Sauer &amp; Thauer (1997, 1998)</td>
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<td>( \Delta G^\circ = -27.5 \text{kJ mol}^{-1} )</td>
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<td>( \text{CH}_2\text{NH}_2^+ + \text{H-S-CoM} \rightarrow \text{CH}_2\text{S-CoM} + \text{NH}_3 )</td>
<td>Monomethylamine : coenzyme M methyltransferase</td>
<td>Burke &amp; Krzycki (1995, 1997); Burke et al. (1998)</td>
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<td>( \Delta G^\circ = -5 \text{kJ mol}^{-1} )</td>
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<td>( \text{CH}_2\text{S-NH}_2 + \text{H-S-CoM} \rightarrow \text{CH}_2\text{S-CoM} + \text{CH}_2\text{NH}_3 )</td>
<td>Dimethylamine : coenzyme M methyltransferase (MtbA + MtbB + ?)</td>
<td>Wassenaar et al. (1996, 1998)</td>
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<td>( \Delta G^\circ = -2-5 \text{kJ mol}^{-1} )</td>
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<td>( \text{CH}_2\text{N}^+ + \text{H-S-CoM} \rightarrow \text{CH}_2\text{S-CoM} + \text{CH}_2\text{NH}_3 )</td>
<td>Trimethylamine : coenzyme M methyltransferase</td>
<td>Ferguson &amp; Krzycki (1997); Wassenaar et al. (1996)</td>
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<td>( \Delta G^\circ = -6-3 \text{kJ mol}^{-1} )</td>
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<td>( \text{CH}_2\text{N}^+ + \text{H-S-CoM} \rightarrow \text{CH}_2\text{S-CoM} + \text{CH}_2\text{NH}_3 )</td>
<td>Tetramethylamine : coenzyme M methyltransferase (MtbA/MtaA + MtbBC)†</td>
<td>Asakawa et al. (1998); Tanaka (1994)</td>
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<td>( \Delta G^\circ = ? )</td>
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<td>( \text{CH}_2\text{S-CH}_3 + \text{H-S-CoM} \rightarrow \text{CH}_2\text{S-CoM} + \text{CH}_2\text{S-H} )</td>
<td>Methylthiol : coenzyme M methyltransferase§ (MtsAB)†</td>
<td>Paul &amp; Krzycki (1996); Tallant &amp; Krzycki (1996, 1997)</td>
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<td>( \Delta G^\circ \approx 0 \text{kJ mol}^{-1} )</td>
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* From Weiss et al. (1994).
† For activity in cell extracts the presence of a methyltransferase-activating protein (MAP), reduced ferredoxin and ATP are additionally required (Daas et al., 1993, 1994, 1996a, b). MtaA (= MT2-M), MtbA (= MT2-A), MtqA and MtsA exhibit methylcobalamin : coenzyme M methyltransferase (MT2) activity (Grahame, 1989; Yeliseev et al., 1993; Ferguson et al., 1996; Wassenaar et al., 1996) and show sequence similarity (LeClerc & Grahame, 1996; Harms & Thauer, 1996b; Paul & Krzycki, 1996; Asakawa et al., 1998). MtaA and MtbA are zinc enzymes (Sauer & Thauer, 1997; MtaC, MtqC, MtbC and MtsB have been shown to be corrinoid proteins harbouring 6-hydroxybenzimidazolyl cobamide as prosthetic group.
‡ Obtained from \( \Delta G^\circ \) for methylation to methane with \( \text{H}_2 \) calculated from the free energies of formation from the elements tabulated in Thauer et al. (1977) and from \( \Delta G^\circ \) of \(-85 \text{kJ mol}^{-1} \) for methyl-coenzyme M reduction with \( \text{H}_2 \) to \( \text{CH}_4 \) and coenzyme M (Thauer et al., 1993).
§ Also catalyses the methylation of coenzyme M with methylmercaptopyropropanol (95%), methylmercaptopropionate (80%), mercaptopropanol (10%), methylmercaptoethanol (8%) and methylacetate (170%) (Tallant & Krzycki, 1997) and the methylation of mercaptoethanol (27%) and 2-mercaptoethanol (66%) (Tallant & Krzycki, 1996).

### Methyl-coenzyme M reductase, its substrate and inhibitors

Methyl-coenzyme M and coenzyme B are the substrates of methyl-coenzyme M reductase, methane and heterodisulphide its products (Bobik et al., 1987; Ellermann et al., 1988) (Fig. 4). From the methyl-coenzyme M analogues tested in cell extracts of *Methanobacterium thermoautotrophicum*, only 2-(methylthio)propanoate (\( K_m = 1-3 \text{mM} \); \( V_{\text{max}} = 1-3 \text{mU mg}^{-1} \)), ethyl-coenzyme M (\( K_m = 1-3 \text{mM} \); \( V_{\text{max}} = 7-4 \text{mU mg}^{-1} \)), methly-seleno-coenzyme M (\( K_m = 0-3 \text{mM} \); \( V_{\text{max}} = 35 \text{mU mg}^{-1} \)) and difluoromethyl-coenzyme M (\( K_m = 2-5 \text{mM} \); \( V_{\text{max}} = 20 \text{mU mg}^{-1} \)) were reduced (methyl-coenzyme M: \( K_m = 0-1 \text{mM} \); \( V_{\text{max}} = 11 \text{mU mg}^{-1} \)) (Wackett et al., 1987). Interestingly, trifluoromethyl-coenzyme M and trifluoromethyl-seleno-coenzyme M (Wackett et al., 1987) and 2-(dimethylsulphonium)ethanesulphonate (Gunsalus et al., 1978) were not reduced, neither were 2-(methylthio)ethanesulphonate, propyl-coenzyme M and 3-(methylthio)propanesulphonate; these compounds were even inhibitors (Gunsalus et al., 1978; Wackett et al., 1987). Other inhibitors based on the methyl-coenzyme M structure are 2-bromoethanesulphonate, ethyl-coenzyme M (\( K_i = 8 \mu\text{M} \)) and 2-chloroethanesulphonate (\( K_i = 70 \mu\text{M} \)) (Gunsalus et al., 1978), 3-bromopropanesulphonate (\( K_i = 50 \mu\text{M} \)), 4-bromobutanesulphonate (\( K_i = 6 \mu\text{M} \)) and 2-azidoethanesulphonate (\( K_i = 1 \mu\text{M} \)).
Methyl-coenzyme M reductase and of its isoenzyme

Methyl-coenzyme M reductase (MCR) has an apparent molecular mass of approximately 300 kDa. It is composed of three different subunits, \( \alpha \) (McrA), \( \beta \) (McrB) and \( \gamma \) (McrG), in an \( \alpha_2\beta_2\gamma_2 \) arrangement (Günsalas & Wolfe, 1980). The enzyme contains 2 mol of tightly but not covalently bound coenzyme \( F_{430} \) mol\(^{-1} \) (Ellenson et al., 1982), which is a nickel porphino, the structure of which is shown in Fig. 5. The enzyme as purified generally also contains 2 mol \( H-S-CoM \) (Hartzell et al., 1987) and 2 mol \( H-S-CO-B \) (Noll & Wolfe, 1986). The enzyme can be reconstituted from its subunits in the presence of coenzyme \( F_{430} \) albeit with very low specific activity (Hartzell & Wolfe, 1986).

*Fig. 4. Structures of methyl-coenzyme M (Taylor & Wolfe, 1974a, b), coenzyme B (Noll et al., 1986) and of the heterodisulfide CoM-S-S-COB (Ellermann et al., 1988). For biosynthesis of coenzyme M and coenzyme B, see White & Zhou (1993), White (1994) and Howell et al. (1998).*

*Fig. 5. Structure of coenzyme \( F_{430} \) in the Ni(II) oxidation state (Pfalz et al., 1982; Farber et al., 1991). For the biosynthesis of coenzyme \( F_{430} \) see Thauer & Bonacker (1994).*

Methanobacterium thermoautotrophicum, Methanothermus fervidus and Methanococcus jannaschii (probably all members of the Methanobacteriales and Methanococcales) have been shown to contain besides MCR a genetically distinct isoenzyme of methyl-coenzyme M reductase designated MRT (Rospert et al., 1990; Brenner et al., 1993; Lehmacher & Klenk, 1994; Bult et al., 1996; Nolling et al., 1996). The expression of the two isoenzymes is differentially regulated by the growth conditions (Bonacker et al., 1992; Pihl et al., 1994; Reeve et al., 1997; Pennings et al., 1997).

The genes encoding the three subunits of the isoenzyme I (MCR) form a transcription unit (mcrBDCGA) which additionally contains two open reading frames encoding two polypeptides, McrC and McrD, of molecular masses below 20 kDa (Cram et al., 1987; Bokranz & Klein, 1991).
Coenzyme F₄₃₀, the prosthetic group of methyl-coenzyme M reductase

The first report on coenzyme F₄₃₀ (Fig. 5) is by Gunsalus & Wolfe (1978), who observed in cell extracts of Methanobacterium thermoautotrophicum a low-molecular-mass non-fluorescent compound with an absorption maximum at 430 nm. In 1979, Schonheit and others discovered that growth of methanogens is dependent on nickel. Diekert et al. (1980a) and Whitman & Wolfe (1980) then showed that the nickel was in part required for the synthesis of F₄₃₀, in which it was incorporated, and that F₄₃₀ contained mol⁻² 1 mol nickel. In the same year, biosynthetic evidence was provided by Diekert et al. (1980b) that F₄₃₀ is a nickel tetrapyrrole, the structure of which was subsequently elucidated in collaboration with the group of A. Eschenmoser at the ETH Zürich (Pfaltz et al., 1982; Färber et al., 1991). Finally, in 1982 it was found that F₄₃₀ is the prosthetic group of methyl-coenzyme M reductase (Ellefson et al., 1982). Until now, coenzyme F₄₃₀ has been found only in methanogenic archaea in which it is always present (Diekert et al., 1981) and in which its only function appears to be the prosthetic group of methyl-coenzyme M reductase.

The presence of a ligand nickel atom is the striking feature of coenzyme F₄₃₀. Nickel brings up to five different metals found in the centre of biological cyclic tetrapyrroles. Up to the discovery of F₄₃₀, the different metals known to occur were iron in haems and in sirohaem, magnesium in chlorophylls, cobalt in corrinoids and copper in turacin, the pigment of turaco bird feathers. Another striking feature is the state of reduction of the pyrrole ring system. One encounters the interesting fact, possibly related to its anaerobic evolution and function, that coenzyme F₄₃₀ is by far the most highly reduced or saturated amongst all known tetrapyrroles (Fig. 6). Coenzyme F₄₃₀ has only five double bonds, of which two pairs are conjugated, but they are separated by two single bonds and so do not form a larger conjugated bond system. The yellow colour of coenzyme F₄₃₀ in contrast to the red colour of porphyrins and corrinoids, is no doubt related to this low degree of conjugation (Friedmann et al., 1991).

Biosynthetically, coenzyme F₄₃₀ is derived from dihydro-sirohydrochlorin, which is also the biosynthetic precursor of sirohaem and B₅₂ (Thauer & Bonacker, 1994; Hungerer et al., 1996).

Coenzyme F₄₃₀, properties relevant to the catalytic mechanism of methyl-coenzyme M reductase

Coenzyme F₄₃₀ is released from methyl-coenzyme M reductase upon denaturation with perchloric acid or trichloroacetic acid. In the released coenzyme, nickel is in the Ni(II) oxidation state. For the understanding of the function of coenzyme F₄₃₀ in methyl-coenzyme M reductase, the following properties of the non-protein-bound cofactor are considered to be of importance (Jaun, 1993; Won et al., 1993; Telser, 1998; see also Stolzenberg & Zhang, 1997; Pandiyan et al., 1997; Schmid et al., 1996, 1997; Jäger & Rudolph, 1997). They are discussed in comparison to those of cobalamin (B₅₂) (Kräutler et al., 1998) (Fig. 7).

F₄₃₀ as isolated in the Ni(II) oxidation state can be reduced to the Ni(I) oxidation state. In aqueous solution, the standard redox potential $E^\circ$ of the Ni(II)/Ni(I) couple is between −600 mV and −700 mV (Jaun & Pfaltz, 1986; Holliger et al., 1993) and is thus in the same
Ni(II) in $F_{430}$ can be methylated with methyl iodide, methyltosylate or methylsulphonium ions (but not with methylthioethers) (Jaun & Pfaltz, 1988; Lin & Jaun, 1992), yielding a methyl-Ni(II) metalloorganic compound (Lin & Jaun, 1991). With methyltosylate, primarily methyl-Ni(III) $F_{430}$ must be formed (Jaun, 1993), just as methylcob(III)alamin is formed upon methylation of cob(1)alamin. Methyl-Ni(III) $F_{430}$ is, however, a very labile compound which is predicted to be almost instantaneously reduced to methyl-Ni(I) $F_{430}$ by any electron donor available (Jaun, 1993). Based on the $E'_0$ of the Ni(III) $F_{430}$/Ni(II) $F_{430}$ redox couple (Jaun, 1990), the $E'_0$ of the methyl-Ni(III)/methyl-Ni(II) couple is estimated to be much more positive than 0 V. In this property, $F_{430}$ differs significantly from cobalamin, the methylated form of which [methylcob(III)alamin] is stable and resistant towards reduction to methyl-

Methyl-Ni(II) $F_{430}$ spontaneously protonolyses to yield CH$_4$ and Ni(II) $F_{430}$ (Lin & Jaun, 1991). It is an electrophilic substitution which is predicted to proceed with retention of stereoconfiguration. To the contrary, methylcob(II)alamin dissociates to a methyl radical and cob(II)alamin rather than to protonolysis to methane and cob(II)alamin.

These properties of $F_{430}$ indicate that the nickel porphyrinoid is a good methyl group reduction catalyst and differs in this property from cobalamin, which is a good methyl transfer catalyst. They are probably relevant to the catalytic mechanism of methyl-coenzyme M reductase: there is evidence that the enzyme only becomes active upon reduction of its prosthetic group from the Ni(II) to the Ni(I) oxidation state (Goubeaud et al., 1997). Furthermore, there is evidence that methyl-coenzyme M reductase catalyses the reduction of the methyl group of methyl-coenzyme M to methane with inversion of stereoconfiguration, consistent with Ni(II) $F_{430}$ methylation and methyl-Ni(II) $F_{430}$ protonolysis as steps in the catalytic cycle (Ahn et al., 1991).

**Activation of methyl-coenzyme M reductase by reduction to the Ni(II) oxidation state**

Methyl-coenzyme M reductase isolated from cells of methanogenic archaea is more or less inactive (Gunsalus & Wolfe, 1980; Brenner et al., 1993). The enzyme purified from *Methanobacterium thermoautotrophicum* ΔH requires the addition of at least two proteins, designated A2 and A3a, of ATP and of Ti(III) citrate for activation in vitro. Even in their presence, however, only less than 0.1% of the specific activity in vivo is reached.

Activation of methyl-coenzyme M reductase by reduction to the Ni(II) oxidation state

MCR (inactive) $\rightarrow$ MCR (0.1% active)

The sequence of *atw*, the A2-encoding gene, predicts that A2 is a 59 kDa ATP-binding protein related to the ABC family of proteins that participates in energy-dependent transport processes (Kuhner et al., 1993).

It was mentioned above that MCR as isolated in its inactive form contains tightly bound 2 mol coenzyme M (Hartzell et al., 1987). When in the presence of the activating system (A proteins, ATP and an electron donor), the inactive enzyme was incubated with [methyl-$^3$H, $^{25}$S]methyl-coenzyme M and component B, the coenzyme M moiety of methyl-coenzyme M was found to slowly exchange into the enzyme-bound coenzyme M during turnover, 150–160 mol CH$_4$ (mol enzyme)$^{-1}$ having to be formed before the exchange into the 2 mol bound coenzyme M was complete (Hartzell et al., 1987). In the absence of the activating system and of component

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Fig. 7. Properties of protein-free coenzyme $F_{430}$ (a) and of cobalamin (b) explaining why the former is a good methyl group reduction catalyst and cobalamin a good methyl group transfer catalyst. CH$_4$, methyltosylate, methylhalides or methylsulphonium ions (Jaun & Pfaltz, 1988; Lin & Jaun, 1992).
B or in the presence of 2-bromoethanesulphonate, neither an exchange nor methane formation was observed. Apparently exchange was dependent on enzyme turnover. The finding of complete exchange indicates that the MCR molecules present were not all activated at the same time and that they lost activity again after several turnovers. The enzyme purified from the Marburg strain of *Methanobacterium thermoautotrophicum* shows residual activity (0.1 U mg⁻¹) in the absence of A₂, A₃a and ATP (Ankel-Fuchs & Thauer, 1986). In the presence of an appropriate reductant, the specific activity even increases to 1 U mg⁻¹, which is 2% of that expected from *in vivo* data (Ellermann et al., 1989). EPR spectroscopic analysis revealed that these enzyme preparations were essentially EPR silent, as were the cells from which the enzyme was purified. This indicates that the inactive methyl-coenzyme M reductase preparations contained F₄305 in the Ni(II) oxidation state.

A first breakthrough was the finding that the specific activity of methyl-coenzyme M reductase was much higher in extracts prepared from cells of *Methanobacterium thermoautotrophicum* reduced with 100% H₂ prior to harvest. Such reduced cells and the methyl-coenzyme M reductase enriched from such cells exhibited a nickel-based EPR spectrum designated MCRred₁ and the spin concentration of the signal correlated with the specific activity. The highest spin concentration reached was between 10 and 20% and the highest specific activity was between 10 and 20 U mg⁻¹ purified methyl-coenzyme M reductase preparations contained F₄305 in the Ni(II) oxidation state.

The final breakthrough was the finding that purified methyl-coenzyme M reductase in the MCRox₁ state (see below) can be activated to a specific activity of 100 U mg⁻¹ by reduction with Ti(III) citrate at pH 10. The reduced enzyme exhibited an axial MCRred₁ signal with a spin concentration near to 100% (Goubeaud et al., 1997).

\[ \text{MCRox₁ (inactive)} \xrightarrow{\text{Ti(III); pH 10}} \text{MCRred₁ (100% active)} \]

Methyl-coenzyme M reductase with a specific activity of 100 U mg⁻¹ shows an EPR spectrum very similar and a characteristic UV/visible spectrum almost identical to that of non-protein-bound coenzyme F₄305 in the Ni(I) oxidation state (Goubeaud et al., 1997). From this finding, it is concluded that the MCRred₁ signal of methyl-coenzyme M reductase is derived from its Ni(I) reduced prosthetic group. From the correlation of the specific activity with the MCRred₁ spin concentration it is deduced that the prosthetic group of methyl-coenzyme M reductase has to be in the Ni(I) oxidation state for the enzyme to be active.

**EPR-signal-exhibiting forms of methyl-coenzyme M reductase:** MCRred₁, MCRred₂, MCRox₁, and MCRox₂

As already indicated, active methyl-coenzyme M reductase exhibits the EPR spectrum MCRred₁. The signal is induced in cells of *Methanobacterium thermoautotrophicum* growing on 80% H₂/20% CO₂ by switching to gassing with 100% H₂. The axial signal builds up within 10 min. Upon further gassing with 100% H₂, a second methyl-coenzyme M reductase derived signal appears, designated MCRred₂, which is rhombic rather than axial (Albracht et al., 1988; for a Ni(I) macrocyclic complex exhibiting a rhombic EPR spectrum see Suh et al., 1996). Cell extracts of such reduced cells also show a high specific activity and also exhibit the MCRred₂ signal. Upon addition of coenzyme M to the cell extracts, the MCRred₂ signal increases; upon addition of methyl-coenzyme M, the MCRred₂ signal is converted to the MCRred₁ signal (Rospert et al., 1991, 1992).

When gassing of growing *Methanobacterium thermoautotrophicum* cultures is switched from 80% H₂/20% CO₂ to 80% N₂/20% CO₂, a methyl-coenzyme M reductase derived EPR signal is induced which differs significantly from the MCRred₁ and MCRred₂ signals and which was designated MCRox₁ (Albracht et al., 1986, 1988). This signal is also induced when the cultures of *Methanobacterium thermoautotrophicum* growing on 80% H₂/20% CO₂ are supplemented with Na₂S (50 mM) (Becker & Ragsdale, 1998). The methyl-coenzyme M reductase in the MCRox₁ form can be purified. The pure enzyme shows only very low specific activity but can be activated by reduction with Ti(III) citrate at pH 10 to the MCRred₁ form (Goubeaud et al., 1997) (see above).

When cultures of *Methanobacterium thermoautotrophicum* growing on 80% H₂/20% CO₂ are supplemented with Na₂SO₃ (10 mM) or Na₂S₂O₄ (10 mM), a methyl-coenzyme M reductase derived novel EPR signal is induced which is designated MCRox₂. The enzyme in this form can be isolated but is inactive. It cannot be activated by reduction with Ti(III) (Becker & Ragsdale, 1998).

**EPR-silent forms of methyl-coenzyme M reductase:** MCRsilent, MCRred₁-silent, MCRred₂-silent, MCRox₁-silent and MCRox₂-silent

Different EPR-silent forms of methyl-coenzyme M reductase have to be discriminated. These forms all have in common that they show only very little activity and are not susceptible to activation by reduction with Ti(III) at pH 10 to the MCRred₁ form. (Residual activity may be due to the presence of traces of EPR active forms.)

The methyl-coenzyme M form referred to as MCRsilent is the one obtained by isolation from cells of *Meth-
anobacterium thermoautotrophicum harvested without prior gassing with 100% H₂ or 20% CO₂/80% N₂. The forms designated MCRred₁ silent, MCRred₂ silent, MCRox₁ silent and MCRox₂ silent are generated spontaneously from the respective EPR active forms when stored under strictly anaerobic conditions at 4 °C: the spin concentration of the different EPR signals decreases by 50% within only a few hours. In the presence of traces of O₂, the MCRred₁ and MCRred₂ signals are instantaneously quenched (Rospert et al., 1991, 1992) whereas the decay rate of the MCRox signals is similar under anaerobic and aerobic conditions (Goubeaud et al., 1997; Becker & Ragsdale, 1998). The latter finding suggests that the MCRox forms of methyl-coenzyme M reductase could contain F₄₃₀ in the Ni(II) oxidation state. Interestingly, the two MCRox forms exhibit a UV/visible spectrum more closely related to that of Ni(II) F₄₃₀ than to Ni(I) F₄₃₀ or Ni(III) F₄₂₀ (Jaun, 1990, 1993).

Crystal structure of methyl-coenzyme M reductase in the MCRox₁ silent state

First crystals of methyl-coenzyme M reductase were obtained in 1991 by Rospert (1991) for the isoenzyme I from Methanobacterium thermoautotrophicum (Marburg). From this time on the enzyme from various methanogens and from various different preparations was crystallized and tested for defraction. Beginning 1996, the first good crystals were obtained from a highly purified preparation of methyl-coenzyme M reductase isoenzyme I in the MCRox₁ state from Methanobacterium thermoautotrophicum (Marburg strain) (Shima et al., 1997). The crystal structure was solved in the MCRox₁ silent state to 1.4 Å resolution (1 Å = 0.1 nm) (Ermler et al., 1997b; see also Ferry, 1997b; and Cammack, 1997). The primary structure of the enzyme had previously been resolved by Bokranz et al. (1988).

Methyl-coenzyme M reductase in the MCRox₁ silent state contains bound 2 mol coenzyme F₄₃₀, 2 mol reduced coenzyme M and 2 mol reduced coenzyme B as revealed by the X-ray structure.

Overall structure

It is characterized by a series of α helices arranged in a compact form with an ellipsoidal shape of about 120 by 85 by 80 Å. The subunits are mutually tightly associated, as indicated by extended interface areas, particularly between subunits α and α' and subunits β and β'.

Two independent active sites

There are two binding sites, roughly 50 Å apart, for coenzyme F₄₃₀, coenzyme M and coenzyme B, forming two separated structurally identical active sites. In each binding site the three coenzymes are embedded inside a narrow channel, 30 Å long, and at the narrowest part 6 Å in diameter, extending from the protein surface deeply into the interior of the protein complex (see the diagram in Fig. 11). The channel and the coenzyme-binding sites are formed by residues of subunits α, α', β and γ (and equivalently α', α, β and γ'), indicating that one trimer is not sufficient to accomplish the enzymic reaction (Ermler et al., 1997b).

Binding of coenzyme F₄₃₀

F₄₃₀ is bound at the bottom of the 30-Å-long channel such that its tetrapyrrole plane points with its front face (reduced pyrrole rings A, B, C and D clockwise) (see Fig. 5) roughly towards the mouth of the channel, whereas its rear face points to the channel bottom. Thus only the front face is accessible to the substrate. Interestingly, in sulphite reductase the prosthetic group sirohaem, which as F₄₃₀ is biosynthetically derived from dihydrosirohydrochlorin, is only accessible from the rear face (Crané et al., 1995, 1997a, b). The tetrapyrrrole ring of coenzyme F₄₃₀ is bound in a rather flat conformation to the enzyme as predicted for the free coenzyme F₄₃₀ in the hexagonally coordinated Ni(II) state (Färber et al., 1991; Jaun, 1993). The Ni atom present as Ni(II) sits almost exactly in the tetrapyrrrole plane and is coordinated to six ligands arranged in a nearly optimal octahedral configuration. The four equatorially located nitrogen atoms of the tetrapyrrrole ring have distances to the nickel of 2.14 Å for ring A, 2.11 Å for ring B, 2.10 Å for ring C and 1.99 Å for ring D (Fig. 8). As the fifth ligand the side chain oxygen of Gln¹⁴₇ protrudes from a long loop to the rear face of F₄₃₀ and approaches the Ni(II) atom to 2.3 Å (Fig. 9). The 1.45 Å electron density map demonstrates that the oxygen and not the nitrogen of the side chain Gln¹⁴₇ is the axial ligand (Ermler et al., 1997b) as to be expected thermodynamically and as seen also in other proteins (Roach et al., 1997). Gln¹⁴₇ is embedded in the protein matrix such that it will probably not be able to move away when F₄₃₀ is reduced from the Ni(II) to the Ni(I) oxidation state in which the Ni(I) is preferentially tetraplanarly coordinated (Jaun, 1993; Telser et al., 1997). By the enforced axial fifth ligand the nucleophlicity of the Ni(I) in F₄₃₀ is predicted to be increased, facilitating the nucleophilic substitution of the methyl group of coenzyme M.

Binding of coenzyme M

In MCRox₁ silent, the sixth coordination site of nickel, located in front of the tetrapyrrrole ring plane of F₄₃₀ is occupied by the thiol group of coenzyme M, which is positioned almost parallel to the tetrapyrrrole plane in contact with its front face (Fig. 9). The thiol group binds axially to the nickel (2.42 Å distance) and interacts with the hydroxyl group of Tyr²₃₀ and Tyr⁵₆₀ and a water molecule that bridges coenzyme M and B. The ethyl moiety is embedded between the lactam ring of the Ni porphinoid and the phenyl ring of Phe₄₂₃. Coenzyme M is anchored to the polypeptide chain by its negatively charged sulphonate group, forming a salt bridge to the
Coenzyme F₄₃₀ in the Ni(II) oxidation state as viewed by looking from outside through the 30-A-long channel into the active site pocket of methyl-coenzyme M reductase (MCR<sub>ox1-silent</sub>). The front face (reduced pyrrole rings A, B, C and D clockwise) is seen. Structure at 1.45 Å resolution.

Fig. 10. Heterodisulphide of coenzyme M and coenzyme B bound via an oxygen of its sulphonate group to Ni(II) in the active site of methyl-coenzyme M reductase (MCR<sub>silent</sub>). Structure at 1.75 Å resolution.

Binding of coenzyme B
With its elongated conformation, coenzyme B fits accurately into the most narrow segment of the channel formed by residues of subunits α, α' and β (see also the diagram in Fig. 11a). Coenzyme B is anchored to the protein mainly by salt bridges between the negatively charged L-threonine phosphate moiety and five positively charged amino acids. The heptanoyl arm is in van der Waal’s contact with several hydrophobic residues. The thiol group of coenzyme B is positioned at a distance of 8.7 Å from the nickel of F<sub>430</sub> and 6.2 Å from the coenzyme M thiol sulphur. It interacts with the side chain of nitrogen of Asn<sup>2481</sup>, the main chain peptide nitrogen of Val<sup>2482</sup> and the bridging water molecule mentioned above. Asn<sup>2481</sup> is within hydrogen bond distance of the sulphur that is presumed to replace the backbone carbonyl oxygen of the modified Gly<sup>2445</sup> (see below).

Five modified amino acids in the active site region
The electron density map revealed five modified amino acids located in subunits α and α’ at or very near the active site region, 1-N-methyl-His<sub>2357</sub>, 4-methyl-Arg<sub>271</sub>, 2-methyl-Gln<sub>200</sub>, S-methyl-Cys<sub>2452</sub> and Gly<sub>2445</sub>, where...
the carbonyl oxygen appears to be substituted by sulphur
(Ermpler et al., 1997b).

**Exclusion of bulked water from the active site**

When coenzyme B is bound to the enzyme the active site is
shielded from bulk solvent. The sole water molecule found in the active site region of MCRox1-silent between
coenzyme M and coenzyme B should be displaced after
binding of the more bulky methyl-coenzyme M. The
active site is lined up by an annular arrangement of Phe330, Tyr333, Phe440, Phe561 and Tyr567 flanked
further by hydrophobic and aromatic residues (Ermpler et al., 1997b). These amino acids are completely
conserved in all methyl-coenzyme M reductases (Nolling et al., 1996). Methane formation from methyl-coenzyme M
and coenzyme B thus takes place in a greasy pocket
and probably does not involve water molecules. These
conditions would allow for radical intermediates. Sol-
vent-inaccessible active sites coated by non-polar aro-
matic residues and attainable by a channel have been
observed in several radical-based enzymes such as
galactose oxidase (Ito et al., 1991), prostaglandin H2
synthase 1 (Picot et al., 1994) and methylmalonyl-CoA
mutase (Mancia et al., 1996).

**Crystal structure of methyl-coenzyme M
reductase in the MCRsilent, MCRred2-silent and
MCRox2-silent states**

The structure of the MCRsilent state was refined in the
resolution range 2.0–10.0 Å with the use of the MCRox1-silent structure for initial phase determination
(Ermpler et al., 1997b). The two enzyme states exhibit
neatly identical overall structures. The model of the
MCRsilent Structure is mainly distinguished from that of
MCRox1-silent by binding of the oxidized instead of the
reduced forms of coenzyme M and coenzyme B (Fig. 10).
A superposition of the structures reveals that the reduced
coenzyme B in MCRox1-silent and the coenzyme B moiety
of the heterodisulphide in MCRsilent align perfectly
except that the sulphur is turned slightly towards
the hydroxyl group of Trp387 and the third to a water
molecule located at the former binding site of the
sulphonate.

The structures of methyl-coenzyme M reductase in the
MCRred2-silent and MCRox2-silent states have, in the
meanwhile, also been resolved but have not yet been
completely refined. Available information indicates that
in the MCRred2-silent state, the active site of methyl-
coenzyme M reductase is probably devoid of both
coenzyme M and coenzyme B. The 30-Å-long channel
leading into the active site pocket is more open and the
residues of the α-, α'-, β- and γ-subunits forming the
channel are more flexible. In the MCRox2-silent state,
methyl-coenzyme M reductase appears to contain bound
coenzyme B but not coenzyme M (W. Grabarse,
unpublished results).

**Catalytic mechanism proposed for methyl-
coenzyme M reductase**

The crystal structures display inactive states of methyl-
coenzyme M reductase with coenzyme F430 in the Ni(II)
oxidation state. Nevertheless, the arrangement of the
coenzymes and the protein environment, combined with
the catalytic properties of the enzyme and the chemical
properties of free coenzyme F430 allow for conclusions
about the active site and the catalytic mechanism (Ermpler et al., 1997b).

First the structure of the enzyme–substrate complex and
of the enzyme–product complex and their formation
and dissociation, respectively, will be discussed. Then
two alternative mechanisms for the conversion of the
enzyme–substrate to the enzyme–product complex will
be proposed.

**Enzyme–substrate/product complex**

In Fig. 11, diagrams of the enzyme–substrate complex
and of the enzyme–product complex are given. The
prosthetic group is shown in the Ni(I) oxidation state.
This is deduced from the finding that methyl-coenzyme
M reductase only exhibits activity when assayed in its
Ni(I) F430 reduced form (either MCRred1 or MCRred2
state) (Rospecht et al., 1991, 1992; Goubeaud et al., 1997).
The diagram in Fig. 11 shows that the prosthetic group
of methyl-coenzyme M is accessible only through a
narrow channel and only by methyl-coenzyme M
(Ermpler et al., 1997b). Through this channel methyl-
coenzyme M must enter before coenzyme B binds since
upon coenzyme B binding the channel is completely
locked. This is consistent with an ordered ternary
complex kinetic mechanism displayed by the enzyme
(Bonacker et al., 1993).

Coenzyme B is able to penetrate the 30-Å-long channel
only with its aliphatic arm and its arm is not long
enough for its thiol group at the end to reach the Ni of
F430. A distance of 8.7 Å remains. A direct reaction of the
coenzyme B thiol group with nickel, as has previously
been proposed (Jaun, 1990; Berkesel, 1991), can there-
fore be excluded. Methyl-coenzyme M is, however,
anchored in the active site to the protein matrix with its
sulphonate group relative to the prosthetic group such
that both the methyl group and the thioether sulphur
could directly interact with the Ni(I). In the
MCRox1-silent structure, coenzyme M probably mimics
the binding position of methyl-coenzyme M with respect
to the binding mode of the sulphonate moiety but
presumably not with respect to the binding mode of the thiol group. A Ni-S-CoM intermediate in the catalytic cycle is not attractive because of the long distance of 6.2 Å between the sulphurs of coenzyme M and coenzyme B. Model building studies indicate, however, that the two sulphurs of coenzyme M and coenzyme B come in van der Waal’s contact when the methyl group of methyl-coenzyme M is placed in van der Waal’s distance of the potentially attacking nickel (Fig. 11a) (Ermler et al., 1997b). Therefore, a Ni-CH₃ intermediate proposed from free coenzyme F₄₃₀ studies (Lin & Jaun, 1991; Jaun, 1993) appears to be compatible with the steric requirements of the active site.

From the structure of the enzyme-substrate complex shown in Fig. 11(a) it is evident that the H⁺ required for methane formation from methyl-coenzyme M is lastly provided by coenzyme B. The MCRoxl-silent structure reveals an interaction between the thiol group of coenzyme B and two hydrogen donors, the amide and peptide nitrogen of Asn₁₄₁ and Val₄₈₂, which would facilitate the cleavage of the H⁺ and permit the presence of a thiolate anion. However, the coenzyme B sulphur is probably too far away from the methyl group of methyl-coenzyme M for direct hydrogen transfer. Tyr₃₃₃ and Tyr₄₆₇ are positioned in the active site such that they could mediate this transfer (Ermler et al., 1997b).

The structure of the enzyme-product complex shown in Fig. 11(b) considers that the heterodisulphide cannot leave the enzyme when the sulphonate oxygen of the coenzyme M moiety binds to the Ni(II) atom of F₄₃₀ as in the MCRoxl-silent state. Coordination of the sulphonate oxygen to nickel is prevented when nickel is present as nucleophilic Ni(I) (Jaun, 1993; Telser et al., 1997). The repulsion between Ni(I) and the sulphonate oxygen might even be used as a driving force to push the heterodisulphide out of the channel. It is not evident at present where, and how the methane gets out (Ermler et al., 1997b).

Both mechanisms of the conversion of the enzyme-substrate complex (Fig. 11a) to the enzyme-product complex (Fig. 11b) in the following to be discussed are based on the assumption that the methyl group of methyl-coenzyme M is transferred to Ni(I) of the prosthetic group before being reduced to methane and that the methyl group of methyl-coenzyme M has to be somehow activated in order that the transfer to Ni(I) F₄₃₀ can proceed (Jaun, 1993).

**Mechanism 1**

It is assumed that methyl transfer from methyl-coenzyme M to Ni(I) yields CH₃-Ni(III) F₄₃₀ involving protonation of methyl-coenzyme M by Tyr₃₃₃ or Tyr₄₆₇ to the sulphonium cation in the transition state. A precedent for such a protonation is found in the crystal structures of ortho-hydroxyphenyl- or ortho-hydroxybenzyl thioethers, giving rise to 5- and 6-membered rings, respectively, but also in the crystal structures of 3,4-dihydro-6-hydroxy-4,5,7,8-pentamethyl-2H-1-(benzo(othio)pyran (d S-H = 2.55 Å, O-H-S = 148 °) (Burton et al., 1985) and 2,3,7,8-tetrahydroxythianthrene (d S-H = 2.53 Å, O-H-S = 167 °) (Mansel & Klar, 1992). The H⁺ would be replenished by the dissociation of coenzyme B.

\[
\text{CH}_3\text{-S-CoM} + \text{H}^+ + \text{Ni(I)} \rightarrow \text{CH}_3\text{-Ni(III) F}_{430} + \text{H-S-CoM} \]

H-S-CoB \rightarrow H^+ + `S-CoB

The CH₃-Ni(III) F₄₃₀ formed by methylation of Ni(I) F₄₃₀ with methyl-coenzyme M is a strong one-electron oxidant and is therefore predicted to oxidize the protonated leaving group to the coenzyme M thyl radical cation.

\[
\text{CH}_3\text{-Ni(III) F}_{430} + \text{H-S-CoM} \rightarrow \text{CH}_3\text{-Ni(II) F}_{430} + \text{H}^+ \text{-S-CoM}^+ \]
Reduction of a thioketone to the corresponding thiol radical with its unpaired electron is a radical and is most thermodynamically feasible.

Mechanism 1 has several features in common with that proposed for ribonucleotide reductase (Lenz & Giese, 1997). An argument against mechanism 1 is that it predicts that methyl-coenzyme M in the absence of coenzyme B should quench the MCRred EPR signal rather than stabilize it (Rospert et al., 1992; Goubeaud et al., 1997).

**Mechanism 2**

One can hypothesize alternative catalytic mechanisms that are consistent with most of the findings. Only one alternative is discussed here. It considers that Ni(I) $F_{430}$ with its unpaired electron is a radical and is most reactive in radical reactions (Jaun, 1993). Methylation of Ni(I) $F_{430}$ with most methyl donors thus directly yields $CH_3-Ni(II)$ $F_{430}$ rather than $CH_3-Ni(III)$ $F_{430}$ (Lin & Jaun, 1991; Jaun, 1993). For methyl-coenzyme M to react with Ni(I) $F_{430}$ in this manner, it has to be activated such that a homolytic cleavage of the S-methyl bond is favoured, e.g. by reaction of methyl-coenzyme M with the coenzyme B thyl radical to the CoM-S-(CH$_3$)-S-CoB sulphuranyl radical (Jaun, 1990; Berkessel, 1991; Tada & Masuzawa, 1997).

The catalytic cycle in the alternative mechanism is therefore assumed to start with the formation of the coenzyme B thyl radical by one electron oxidation of coenzyme B.

$H-S-CoB + X → S-CoB + XH$

The electron acceptor $X$ cannot be Ni(II) $F_{430}$ as has been proposed since the enzyme is only active in its Ni(I) $F_{430}$ reduced form (Goubeaud et al., 1997). Therefore, there must be a second redox active group capable of oxidizing coenzyme B to the coenzyme B thyl radical, and indeed there is evidence for two independent redox active groups in methyl-coenzyme M reductase (Rospert, 1991; Rospert et al., 1992). This group could be the modified Gly$^{2445}$, a thiglycine, which interacts with the thiol group of coenzyme B via the side chain nitrogen of Asn$^{2481}$ and which could be susceptible to reduction to the thioethyl radical $X^-$ (Buckel & Keese, 1995; Buckel, 1996). Probably the protonated thioethyl would be reduced to the protonated thioethyl (XH) since this reaction is energetically more favourable as deduced from the thermodynamics of the reduction of ketones to ketyl in the protonated and unprotonated forms (Lenz & Giese, 1997). The coenzyme B thyl radical would react with methyl-coenzyme M, generating the above-mentioned sulphuranyl radical. From the sulphuranyl radical, the methyl radical would be transferred to Ni(I) $F_{430}$, generating the heterodisulphide and $CH_3-Ni(II)$ $F_{430}$ which would protonolyse to Ni(II) $F_{430}$ and $CH_4$.

$CoM-S(CH_3)-S-CoM + Ni(I) F_{430} → CoM-S-S-CoB + CH_3-Ni(II) F_{430}$

$CH_3-Ni(II) F_{430} + XH → Ni(II) F_{430} + CH_4 + X^-$

The catalytic cycle would be closed by electron transfer from $X^-$ to Ni(II) $F_{430}$.

$X^- + Ni(II) F_{430} → X + Ni(I) F_{430}$

Via endergonic $XH$ dissociation to $X^- + H^+$, the two last reactions could be thermodynamically tightly coupled.

The salient feature of mechanism 2 is that it involves transient methyl radical intermediates and does not involve $CH_3-Ni(II)$ $F_{430}$ as intermediate in the catalytic cycle. Transient methyl radical intermediates could possibly account for the presumed methylation of His$^{2257}$, Arg$^{2271}$, Glu$^{2460}$ and Cys$^{3432}$.

Future studies will have to show which of the proposed catalytic mechanisms is correct or whether both are wrong. Unfortunately, one can only disprove but not prove a catalytic mechanism (Stinson, 1995).

**From hydrogenase to methyl-coenzyme M reductase**

It has been a long way from the first demonstration of an enzyme, of hydrogenase, in methanogens in 1933 by Stephenson & Stickland (1933a) to the crystal structure of methyl-coenzyme M reductase in 1997 and there is still a good way to go until the biochemistry of methanogenesis is completely understood. Many ques-
tions remain to be answered. For methyl-coenzyme M reductase, the most pertinent questions are, what functions do the five amino acid modifications in the enzyme have and how are these modifications brought about? The answers to these questions could be the key to understanding of the catalytic mechanism of the methane-forming enzyme. Fortunately, recently a genetic system for archaea of the genus *Methanosarcina* has become available (Metcalf et al., 1997) now allowing these problems to be also tackled with genetic methods.

The work of my group on the biochemistry of methanogenesis started with growth experiments and the discovery that growth of methanogens is dependent on nickel (Schönheit et al., 1979). This led to the finding that coenzyme F₄₃₀ is a nickel porphinoid (Diekert et al., 1980a, b), that carbon monoxide dehydrogenase from methanogens contains nickel (Hamel et al., 1984) and that the hydrogenases first described by Marjory Stephenson (Stephenson & Stickland, 1931a) are nickel enzymes (Graf & Thauer, 1981; Albracht et al., 1982; Albracht, 1994) (for recent reviews on nickel enzymes including methyl-coenzyme M reductase see Cammack & van Vliet, 1998; Maroney et al., 1998). Later we found that methanogens also contain a metal-free hydrogenase (Zirngibl et al., 1990; Thauer et al., 1996) and just recently that methanogens contain a fourth type of hydrogenase (Künkel et al., 1998) that shows sequence similarity to hydrogenase 3 (Sauer et al., 1992) and 4 (Andrews et al., 1997) of the formate hydrogenlyase from *E. coli*, an enzyme complex first studied in detail in the laboratory of Marjory Stephenson now almost 70 years ago (Stickland, 1929; Stephenson & Stickland, 1932, 1933b; Woods, 1936). With respect to my research, I, therefore, feel myself in the scientific tradition of Marjory Stephenson. And there is even a genealogical connection: I learned to grow methanogens from Greg Zeikus when he was in Marburg for a sabbatical (Zeikus et al., 1977). He himself had obtained his training from Ralph Wolfe in Urbana (Zeikus & Wolfe, 1972), who discovered methyl-coenzyme M reductase (Wolfe, 1991).

The first work of Ralph Wolfe on methanogens is together with Sidney Eldsen (Knight et al., 1966), who had been a student of Marjory Stephenson in the Cambridge Biochemistry Laboratory (Wolfe, 1991) where in 1933 the modern era of methanogenesis began (Wolfe, 1993). I am therefore very much indebted to the Society for General Microbiology for having chosen me to give the 1998 Marjory Stephenson Lecture.

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