Activation Mechanism of Methanol:5-Hydroxybenzimidazoylcobamide Methyltransferase from Methanosarcina barkeri*

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Methanosarcina barkeri can utilize methanol as sole source for methanogenesis and growth. The first step in the reduction of methanol to methane is the formation of an enzyme-bound methylcobamide catalyzed by methanol:5-hydroxybenzimidazoylcobamide methyltransferase (MT$_1$) (1). The methyl group of methylated MT$_1$ is subsequently transferred to 2-mercaptopoethanesulfonic acid in Methanosarcina barkeri. MT$_1$ only binds the methyl group of methanol when the cobalt atom of its corrinoid prosthetic groups is present in the highly reduced Co(I) state. Formation of this reduct state requires H$_2$, hydrogenase, methyltransferase activation protein, and ATP. Optical and electron paramagnetic resonance spectroscopy studies were employed to determine the oxidation states and coordinating ligands of the corrinoids of MT$_1$ during the activation process. Purified MT$_1$ contained 1.7 corrinoids per enzyme with cobalt in the fully oxidized Co(III) state. Water and 3-N of the 5-hydroxybenzimidazoyl base served as the upper and lower ligands, respectively. Reduction to the Co(II) level was accomplished by H$_2$ and hydrogenase. The cob(II)amide of MT$_1$ had the base coordinated at this stage. Subsequent addition of methyltransferase activation protein and ATP resulted in the formation of base-uncoordinated Co(II) MT$_1$. The activation mechanism is discussed within the context of a proposed model and compared to those described for other corrinoid-containing methyl group transferring proteins.

Methanosarcina barkeri utilizes methanol as sole source for methanogenesis and growth. The first step in the reduction of methanol to methane is the formation of an enzyme-bound methylcobamide catalyzed by methanol:5-hydroxybenzimidazoylcobamide methyltransferase (MT$_1$) (1). The methyl group of methylated MT$_1$ is subsequently transferred to 2-mercaptopoethanesulfonic acid (coenzyme M, HS-CoM) by Co-methyl-5-hydroxybenzimidazoylcobamide:HS-CoM methyltransferase (MT$_2$) (2). As a result methyl-coenzyme M (CH$_3$-S-CoM) is produced, which is the substrate for the final step in methanogenesis in all methanogens studied so far (3).

The corrinoid prosthetic group of MT$_1$ can only be methylated by methanol when the central cobalt atom of the cobamide is present in the highly reduced Co(I) state (4, 5). Since this state is extremely sensitive toward oxidation, MT$_1$ readily inactivates upon manipulation and even during catalysis. Reactivation is possible and requires participation of a reducing system, methyltransferase activation protein (MAP), and ATP (4, 6–8). The reducing system consists of hydrogen, hydrogenase, and ferredoxin. Ferredoxin is not absolutely required, though it stimulates the apparent reaction rate of methyl group transfer (6).

Here, we report the UV-visible absorbance and electron paramagnetic resonance properties of the corrinoid prosthetic groups of MT$_1$ under various additions of the reducing system, MAP, and ATP. From these results, the sequence of events leading to the formation of the cob(II)amide of MT$_1$ is deduced. The activation of MT$_1$ proceeds by a novel mechanism, which is presented in a model and compared to those described for other corrinoid-containing methyl group transferring proteins.

Experimental Procedures

Cell Material—Cells of M. barkeri strain MS (DSM 800) were grown and harvested, and cell extract was prepared anaerobically as described previously (6, 9).

Enzyme Assays—Incubation mixtures were prepared in an anaerobic glove box and reactions were performed in crimp-sealed 10-ml serum vials. MT$_1$ activity was determined by measuring the methanol-dependent HS-CoM conversion to CH$_3$-S-CoM when added to a reaction mixture containing MT$_2$/hydrogenase, MAP, and ferredoxin fractions obtained after DEAE-Sepharose fractionation of cell extract of M. barkeri (6). A typical reaction mixture (final volume, 100 µl) contained 50 mM TES/K$_+^+$ buffer (pH 7.0), 24 mM MgCl$_2$, 10 mM methanol, 10 mM HS-CoM, 2 mM ATP, 1 mM 2-bromothanesulfonic acid, 20 µl of MT$_2$/hydrogenase fraction, 5 µl of ferredoxin fraction, 25 µl of MAP fraction, and an amount (usually 25 µl) of MT$_1$ to be tested (6). After gassing with 50% H$_2$/50% N$_2$ (100 kPa), the vials were kept on ice. Reactions were started by placing the vials at 37°C. After appropriate incubation periods, routinely 0, 15, 30, 45, and 60 min, reactions were stopped by placing the vials on ice. Activity of methyl group transfer of methanol to HS-CoM was routinely assayed by measuring the decrease in the amount of HS-CoM (see below). The methyltransferase activity obtained was linearly dependent on the amount of MT$_1$ added.

The enzymatic activities of MT$_2$, MAP, hydrogenase, and ferredoxin were determined as described previously (4, 6, 8).

Protein Purification—Because several of the enzymes involved in the methanol:HS-CoM methyltransferase reaction are oxygen-labile (1, 4, 6, 8), all hadlings were performed in an anaerobic glove box (97.5% N$_2$, 2.5% H$_2$) at room temperature. The purification procedure started by applying 10 ml of cell extract to a DEAE-Sepharose-CL-6B column and separating the proteins involved in the methyltransferase reaction as described previously (6). MT$_2$ and hydrogenase activity eluted between 0.20 and 0.22 M NH$_4$Cl. Fractions of 0.25 M and 0.34 M NH$_4$Cl

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1The abbreviations used are: MT$_1$: methanol:5-hydroxybenzimidazoylcobamide methyltransferase; CH$_3$-S-CoM (methyl-coenzyme M); 2-mercaptoethanesulfonic acid; HS-CoM (coenzyme M), 2-mercaptoethanesulfonic acid; MT$_2$: Co-methyl-5-hydroxybenzimidazoylcobamide:HS-CoM methyltransferase; MAP, methyltransferase activation protein; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; TES, N-tris(hydroxymethyl)methyl-2-aminooethanesulfonic acid; HBI, 5-hydroxybenzimidazoyl; B$_{12}$-HBI, 5-hydroxybenzimidazoylcobamide; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; kPa, kilopascal(s).
Activation Mechanism of Methanol Methyltransferase I

The purification procedure started from 10 ml of cell extract. Enzyme assays were performed as described under "Experimental Procedures." Units are expressed as micromoles of 2-mercaptoethanesulfonic acid methylated per min.

| Step                      | Total protein | Total activity | Specific activity | Factor | Recovery |
|---------------------------|---------------|----------------|-------------------|--------|----------|
| Cell extract              | 260           | 44.20          | 0.17              | 1      | 100      |
| DEAE-Sepharose            | 4.4           | 16.98          | 3.86              | 23     | 58       |
| TSK DEAE                  | 0.6           | 3.34           | 5.57              | 33     | 8        |

Native PAGE, denaturing SDS-PAGE, and isoelectric focusing were performed with prefabricated minigels using the PhastSystem equipment (Uppsala, Sweden). The gels were stained with Coomassie Brilliant Blue R-250. The subunit molecular weight of MT was determined by electrophoresis on a 10–15% gradient minigel with SDS-buffers. The markers (Bio-Rad) were the following (Da): α-lactalbumin (14,400), soybean trypsin inhibitor (21,500), bovine carbonic anhydrase (31,000), hen egg white lysozyme (45,000), bovine serum albumin (66,200), and rabbit muscle phosphorylase b (97,400). Native PAGE was performed on a 5–25% gradient minigel. Isoelectric focusing was performed with a pH 3–9 isoelectric focusing gel using the PI 3.5–9.6 isoelectric focusing standard proteins from Bio-Rad.

Protein was determined with the Bio-Rad protein reagent with bovine serum albumin as a standard. Molar concentrations of MT were calculated from the molecular mass (122,000 Da) of the protein (1). Molar amounts of hydrogen apatite-purified MAP were estimated from the reported Mₚ = 60,000 of the protein, taking into account that approximately one-third of the total protein was MAP (8). Corrinoids were quantified after conversion into the dicyanocobamide derivatives. Samples of MT were diluted in 50 mM CAPS buffer (pH 10) containing 5 mM potassium cyanide and incubated for 5 min at 90°C (14). Concentrations were calculated from the absorption at 580 nm (ε₅₈₀ = 10.2 × 10⁶ M⁻¹ cm⁻¹) (15). High performance liquid chromatography analysis of corrinoids was performed as described before (6). HS-CoM was measured by the method of Ellman (16). Samples of 25 μl were mixed with 3 ml 0.48 mM 2,2’-dinitro-5,5’-dithiobenzoic acid in 150 mM Tris/Cl buffer (pH 8.0) and immediately measured at 412 nm. Total iron was determined as described by Fish (17). Manganeses was measured as described by Bartley and co-workers (18). The effect of bathophenanthroline disulfonate was tested anaerobically essentially as described by Rouverie and Wolfe (19).

Materials—All chemicals used were of analytical grade. HS-CoM, 2-bromoethanesulfonic acid, TES, CAPS, bathophenanthroline disulfonate, and hexokinase (yeast type VI) were purchased from Sigma. Dithiothreitol was from Serva Feinbiochemica (Heidelberg, Germany). ATP, CHAPS, and myokinase (adenylate kinase) were purchased from Boehringer (Mannheim, Germany). DEAE-Sepharose-CL-6B was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). TSK DEAE-5-PW and TSK-Gel HA-1000 columns were obtained from TosohHaas (Stuttgart, Germany). Sep-Pak CM cartridges were acquired from Waters Associates (Milford, MA). Gases were supplied by Hoek-Loois (Schiedam, The Netherlands). To remove traces of oxygen, H₂-containing gases were passed over a BASF RO-20 catalyst at room temperature; nitrogen was passed over a prerduced BASF R3–11 catalyst at 150°C. The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany).
determined from three separate purifications. We found that
the presence of Mg$^{2+}$ (15 mM) in the buffer systems was a
prerequisite in the isolation of MT1. Treatment of MT1 prepa-
rations with 50 mM EDTA, which removed Mg$^{2+}$, and subse-
quent native PAGE showed the loss of the corrinoid prosthetic
group and a dissociation of the protein (not shown). Appar-
ently, Mg$^{2+}$ plays a role in binding of the corrinoid prosthetic
group and in subunit association. From isoelectric focusing, a
pI of 4.5 was obtained. Determination of non-heme iron re-
vealed the presence of 1.7 mol of Fe/mol of MT1; acid-labile
sulfur could not be detected. Pretreatment with 2 mM of the
ironchelator bathophenanthrolinedisulfonate did not result in
any loss of activity. In addition, EPR spectroscopy studies of
MT1, at 14 K, in the absence and presence of 2 mM dithionite,
did not reveal any signals indicative of the presence of iron-
sulfur clusters. From this, it was concluded that MT1 does not
contain functionally active iron.

UV-visible Spectroscopy—MT1 was anaerobically isolated as
a red-colored protein. The UV-visible spectrum of the pros-
thetic group (Fig. 2) was typical of Co(III) corrinoids (20–22).
Exposure to air did not further alter the spectrum. The pros-
thetic group showed absorption maxima at 352 (2.89), 493
(1.07), and 522 (1.04) nm with shoulders at 388 (1.16), 403
(1.04), and 465 (1.0) nm (the numbers in parentheses express
the absorbance relative to that of the shoulder at 465 nm). The
major visible absorption band at 352 nm is characteristic of the
presence of water as the upper ligand (21, 22). Compared to
free aquo-B$_{12}$-HBI, the absorbance of the corrinoid of MT1 was
relatively increased in the 375–475 nm region. Incubation of
purified cob(III)amide-MT1 with up to equimolar amounts of
MAP and/or ATP (up to 5 mM) had no effect on the UV-visible
spectrum of the corrinoid prosthetic groups.

When MT1 was incubated under hydrogen with a small
amount of MT$_2$/hydrogenase and ferredoxin, the protein turned
yellow. The spectrum (Fig. 3) displayed the typical character-
istics of cob(II)amide (5, 20, 21). Absorption maxima were ob-
served at 417 (1.31) and 465 (1.29) nm with shoulders at 315
(3.64), 356 (2.11), and 535 (1.0) nm (the numbers in parenthe-
ses indicate the absorbance relative to that of the shoulder at 535 nm). Compared to the spectrum of free aquo-B$_{12}$-HBI, the absorbance of the corrinoid of MT1 was relat-
ively increased in the 375–475 nm region. Incubation of
purified cob(III)amide-MT1 with up to equimolar amounts of
MAP and/or ATP (up to 5 mM) had no effect on the UV-visible
spectrum of the corrinoid prosthetic groups.

The spectrum of the hydrogen-reduced Co(II) form of MT1
was not altered by the subsequent addition of MAP, ATP, or
methanol alone. When both MAP and ATP were added the
shoulder at 315 nm of the cob(II)amide of MT1 disappeared
while no significant changes occurred at higher wavelengths
(Fig. 3). Interestingly, a distortion of the absorbance band at
315 nm is the major feature, when the HBI nucleotide in free
Co(II) B$_{12}$-HBI becomes decoordinated ("base-off") upon acidi-
The spectrum could be simulated on the basis of a near-axial, "base-on" form of Co(II) corrinoids (Fig. 4A) was observed (23). When MT1 was incubated in the presence of the reducing system (H2, hydrogenase, ferredoxin), MAP and ATP, we never detected the characteristic absorption peak of cob(I)amide at 390 nm. However, the additional presence of metha-

**DISCUSSION**

Optical and EPR spectroscopical studies were employed to determine the oxidation state of the central cobalt atom and the coordination of the ligands in the corrinoid protein MT1 under various additions of MAP, ATP, and a reducing system. After isolation MT1 contained somewhat less than 2 mol of B12-HBI/mol of αβ protein. The UV-visible light spectrum (Fig. 2) indicated that the prosthetic group was present in the hexacoordinated Co(III) oxidation state with the nucleotide 5-hydroxybenzimidazole and water as the lower and upper ligands, respectively. Purified MT1 is inactive, and reactivation apparently requires the reduction of Co(III), which is brought about by a reducing system (H2, hydrogenase, and ferredoxin), and MAP, and ATP (4, 6, 8) (Fig. 5). Incubation of MT1 with hydrogen, hydrogenase, and ferredoxin resulted in the reduction to the Co(II) state. EPR spectroscopy demonstrated that the HBI-base was still coordinated at this stage (base-on). Subsequent addition of both MAP and ATP induced a conversion of base-on
into base-off Co(II) MT₁₁. We previously showed MAP to be autophosphorylated by ATP; MAP-phosphate is able to substitute for the requirement of MAP and ATP (8). The phosphorylated protein, thus effects MT₁₁ in such a way that the HBI-base becomes dissociated. In the experiment shown in Fig. 4, an estimated 38% of the corrinoids became base-uncoordinated when MT₁ (15.8 μM) was incubated with 7.5 μM MAP and excess ATP (5 mM). From this, it follows that the concentration of base-off MT₁ amounted to 6.0 μM, which is about equimolar with respect to MAP added. In agreement with this conclusion, lower amounts of base-off cob(II)amidewereobtained when the MAP concentration was decreased in the EPR experiments.

The conversion of base-on into base-off cob(II) amide has an important implication. In non-protein bound B₁₂-HBI, such base-off conversion causes the midpoint redox potential of the Co(II)/Co(I) couple to increase from -592 to -500 mV (21). In a similar way, the action of MAP and ATP may facilitate the reduction in MT₁ of cob(II)amide to the catalytically active species, the powerful nucleophile cob(Ⅰ)amide. Although we could not detect the direct formation of cob(Ⅰ)amide in our experiments, the findings that (i) the species is produced upon demethylation of methylated MT₁ (5) and (ii) methyl-B₁₂-HBI bound to MT₁ is formed during the activation of the enzyme in the presence of H₂, hydrogenase, MAP, ATP and methanol (this paper; Refs. 4–6), demonstrate that cob(Ⅰ)amide must play a role in the catalytic and reductive activation cycles. The cob(Ⅰ)amide/cob(Ⅰ)amide midpoint redox potentials are strongly influenced by the protein environment. In the corrinoid/iron-sulfur proteins involved in acetyl-CoA synthesis and degradation that have been isolated from Clostridium thermoceticum (24) and from Methanosarcina thermophila (25), reduction to the catalytically active Co(I) state occurred at midpoint redox potentials of −504 mV (26) and −486 mV (25), respectively. Here, reduction has to be performed by electrons derived from the carboxyl (CO)/CO₂ oxidation (E₀' = −520 mV). It is important to note that the enzymes do not require ATP for the activation; in the purified, inactive cob(II)amide state, the corrinoids are already contained in the (at neutral pH) thermodynamically unfavorable base-off state by the protein backbone. Here, the observed midpoint redox potentials about equaled the E₀' = −500 mV of the free base-off cob(II)amide/cob(Ⅰ)amide couple. For a number of corrinoid-containing methyltransferases, midpoint potentials have been measured that were significantly higher than found for the corresponding B₁₂ derivatives in solution (25–28). For example, the reduction of base-off cob(II)amide to cob(Ⅰ)amide in the membrane-bound methyltetrahydrodemanthoeprotein:HS-CoM methyltransferase complex from Methanosarcina mazei showed an E₀' as high as −426 to −450 mV, which is about 150 mV more positive than the analogous reduction of free B₁₂-HBI (28).

In comparing the UV-visible light spectra of MT₁ with aqueous solutions of B₁₂-HBI, we noticed some differences in the 400 nm and 500–600 nm regions (Figs. 2 and 3). Since MT₁ does not contain Fe-S clusters (this paper) or other chromophoric groups (results not shown), this had to be caused by a conformational distortion of the corrin ring structure by the protein (29). Such distortion is likely to change the reduction potential of the prosthetic group. Incubation of methyltetrahydrodemanthoeprotein:HS-CoM methyltransferase with ATP and the methyl donor (methyltetrahydrodemanthoeprotein) raised the apparent midpoint potential another 200 mV (E₀' = −245 mV) (28), i.e. to a level where reduction becomes feasible at even very low hydrogen concentrations (E₀' = −414 mV). Remarkably, the 200 mV shift was not observed when the methyl donor was omitted, and the authors (28) proposed that the simultaneous action of ATP and the methylating substrate in a ternary enzyme complex is required for raising the redox potential. This may also apply to MT₁₁. As pointed out above, we never observed the characteristic UV-visible light spectral features of cob(Ⅰ)amide upon incubation under even high (100 kPa) hydrogen partial pressure of MT₁₁ with MAP and ATP. However, the additional presence of methanol results in the formation of methyl-B₁₂-HBI in MT₁, suggesting a cooperative action of MAP-phosphate and methanol in the reductive activation (Fig. 5). The EPR experiments outlined above indicated that the amount of base-off cob(Ⅰ) amide formed was dependent on the amount of MAP added. Yet, MT₁₁ may be fully activated in the presence of substoichiometric amounts of MAP provided ATP and methanol is present (6, 8). This may be explained by assuming that MAP becomes dephosphorylated after completion of the activation cycle of the MT₁₁ molecule (8) (Fig. 5). Rephosphorylation by ATP then yields MAP-phosphate for activation of another molecule. Future investigations have to clarify questions with respect to the corrinoid midpoint redox potentials in MT₁₁ and the effects hereon of MAP-phosphate and methanol.

In order to be active, corrinoid-dependent methyltransferases often require an ATP-dependent reductive activation (3, 30). As yet, only the activation mechanism of methionine synthase has been elucidated (31). Here, ATP is the substrate in the formation of the potent methylating agent, S-adenosyl methionine, which traps Co(I) out of the thermodynamic unfavorable Co(III)/Co(I) reduction equilibrium (31). In this paper, we have presented evidence that nature developed another approach to facilitate the generation of the active enzymes, notably by inducing in an ATP-dependent process the conformational change of the prosthetic group. Perhaps other corri-
noid-containing methyltransferases from methanogens (3) and other obligate anaerobic organisms (32) are activated in a similar fashion.

Acknowledgments—We thank Jeroen Zandbergen and Steven van den Berg for assistance in the purification and characterization of MT 1. We gratefully acknowledge Roel Wassenaar for skillful determination of the effect of MAP-phosphate.

REFERENCES
1. van der Meijden, P., te Brömmelstroet, B. W., Poirot, C. M., van der Drift, C., and Vogels, G. D. (1984) *J. Bacteriol* **160**, 629–635
2. vanderMeijden, P., Heythuysen, H. J., Pouwels, A., Houwen, F., van der Drift, C., and Vogels, G. D. (1983) *Arch. Microbiol.* **134**, 238–242
3. Keltjens, J. T., and van der Drift, C. (1986) *FEMS Microbiol. Rev.* **39**, 259–303
4. van der Meijden, P., van der Lest, C., van der Drift, C., and Vogels, G. D. (1984) *Biochem. Biophys. Res. Commun.* **118**, 760–766
5. van der Meijden, P., Jansen, L. P. J. M., van der Drift, C., and Vogels, G. D. (1983) *FEMS Microbiol. Lett.* **19**, 247–251
6. Daas, P. J. H., Gerrits, K. A. A., Keltjens, J. T., van der Drift, C., and Vogels, G. D. (1993) *J. Bacteriol.* **175**, 1278–1283
7. van der Meijden, P., Heythuysen, H. J., Sliepenbeek, H. T., Houwen, F. P., van der Drift, C., and Vogels, G. D. (1983) *J. Bacteriol.* **153**, 6–11
8. Daas, P. J. H., Wassenaar, R. W., Willemsen, P., Theunissen, R. J., Keltjens, J. T., van der Drift, C., and Vogels, G. D. (1996) *J. Biol. Chem.* **272**, 22339–22345
9. Hutten, T. J. De Jong, M. H., Peeters, B. P. H., van der Drift, C., and Vogels, G. D. (1981) *Biochem. Biophys. Res. Commun.* **108**, 731–737
10. Pierik, A. J., and Hagen, W. R. (1991) *Eur. J. Biochem.* **202**, 1291–1297
11. Holliger, C., Pierik, A. J., Reijerse, E. J., and Hagen, W. R. (1993) *J. Am. Chem. Soc.* **115**, 5651–5656
12. Arendsen, A. F., Verhagen, M. F. J. M., Wolbert, R. G. B., Pierik, A. J., Stams, A. J. M., Jetten, M. S. M., and Hagen, W. R. (1993) *Biochemistry* **32**, 10323–10330
13. Ljungdahl, L. G., LeGall, J., and Lee, J.-P. (1973) *J. Biol. Chem.* **12**, 1802–1808
14. Pol, A., van der Drift, C., and Vogels, G. D. (1982) *Biochem. Biophys. Res. Commun.* **108**, 731–737
15. Eilman, G. L. (1958) *Arch. Biochem. Biophys.* **74**, 443–450
16. Fish, W. W. (1968) *Methods Enzymol.* **156**, 357–364
17. Harder, S. A., Lu, W.-P., Ragsdale, S. W., and Ferry, J. G. (1993) *J. Biol. Chem.* **268**, 325–329
18. Banerjee, R. V., and Matthews, R. G. (1990) *Biochemistry* **29**, 1129–1135
19. Lu, W.-P., Becher, B., Gottschalk, G., and Ragsdale, S. W. (1995) *J. Bacteriol.* **177**, 2245–2250
20. Gianotti, C. (1982) in *B12* (Dolphin, D., ed) Vol. 1, pp. 393–430, John Wiley & Sons, New York
21. Banerjee, R. V., and Matthews, R. G. (1990) *FASEB J.* **4**, 1450–1459
22. Diekert, G., and Wolkhardt, G. (1994) *Antonie van Leeuwenhoek* **66**, 209–221