Components Required for the Formation of CH$_4$ from Methylcobalamin by Extracts of *Methanobacillus omelianskii*

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**ABSTRACT**

Wood, J. M. (University of Illinois, Urbana), and R. S. Wolfe. Components required for the formation of CH$_4$ from methylcobalamin by extracts of *Methanobacillus omelianskii*. J. Bacteriol. 92:696-700. 1966.—Optimal conditions for the formation of CH$_4$ from methylcobalamin (methyl-coA, 5β,6β-dimethylbenzimidazolyldcobamide) by partially purified extracts of *Methanobacillus omelianskii* (*Methanobacterium omelianskii*) were achieved by the addition of two protein fractions, adenosine triphosphate, Mg$_2^+$, and a reduced flavin adenine dinucleotide-generating system. Adenosine diphosphate and adenosine monophosphate play an important role in the regulation of C$_4$ transfer in this reaction. The possible position of adenosine triphosphate in B$_12$-dependent methyl transfer is discussed.

Methylcobalamin has received attention recently, since it serves as a methyl-donor in several transmethylation reactions. Blaylock and Stadtmann (1) first demonstrated that it was an excellent substrate for the formation of CH$_4$ by extracts of *Methanosarcina barkeri*, and Wolin, Wolin, and Wolfe (9) obtained similar results when they demonstrated the adenosine triphosphate (ATP)-dependent formation of CH$_4$ with crude extracts of *Methanobacillus omelianskii* (*Methanobacterium omelianskii*).

Because of the instability of the methane-forming system, no cofactors other than ATP could be demonstrated as requirements for this reaction at that time. However, we have developed a technique for the partial purification of the methane-forming system of *M. omelianskii* which enables elucidation of its cofactor requirements. Particular attention is focused on the requirement for ATP, since this cofactor has been implicated not only in methane formation, but also in methyl transfer for B$_12$-dependent methionine biosynthesis in *Escherichia coli* (3), and in the synthesis of the methyl group of acetate from methylcobalamin in extracts of *Clostridium thermoacetemicum* (6).

**MATERIALS AND METHODS**

**Culture methods.** *M. omelianskii*, obtained from H. A. Barker, was mass-cultured as outlined previously (8). Larger crops of cells were obtained by inoculating 180 liters of medium, contained in a stainless-steel drum, with 40 liters of an actively growing culture.

**Preparation of cell-free extracts.** Cells were harvested in a Sharples centrifuge, and crude extracts were prepared by exposing these bacteria, 1 g (wet weight) per ml of 0.5 M potassium phosphate buffer at pH 7.0, to the maximal output of a Branson sonic probe for 2 min at 0 C. Cell debris was removed by centrifugation at 23,000 × g for 20 min at 0 C.

**Sephadex treatment (component A).** Compounds of low molecular weight were removed from crude extracts by passage down a Sephadex G 25 column.

**Acetone fractionation (component B).** Crude extracts were heated to 60 C for 6 min, followed by centrifugation at 23,000 × g for 10 min at 0 C. The supernatant solution from these heated extracts was then treated with a solution of protamine sulfate (pH 5.0) to a saturation of 1.5 mg of protamine sulfate per 10 mg of protein. This procedure was followed by centrifugation at 23,000 × g for 10 min at 0 C. The supernatant solution from protamine sulfate treatment was fractionated with acetone at −10 C. The fraction which was precipitated at 45 to 65% dilution was retained and dissolved in 0.05 M potassium phosphate buffer at pH 7.0. All centrifugation steps were carried out at −10 C during acetone fractionation. Protein was determined by the biuret procedure (2).

**Enzymatic assays.** Methane was assayed by the gas chromatographic technique described previously (8); all reactions were performed under hydrogen atmosphere at 40 C. ATP content was assayed by use of a Mannheim and Boehringer ATP-kit (Calbiochem). Generation of reduced flavin adenine dinucleotide (FADH$_2$) was accomplished by adding 0.2 μmol of...
nicotinamide adenine dinucleotide (NAD), 0.04 
µmole of flavin adenine dinucleotide (FAD), and 40.0 
µmole of ethyl alcohol to each reaction flask. Since 
*M. omelianskii* was grown on ethyl alcohol, extracts 
contained an active alcohol dehydrogenase which 
served as a primary source of electrons for FADH₂ 
generation.

**Materials.** Methylcobalamin was synthesized as 
described previously (10). B₃₈-coenzyme (5,6- 
dimethylbenzimidazolyloxylic acid coenzyme) was syn-
thetized by the method of Johnson et al. (4). We 
are indebted to H. P. C. Hogenkamp for providing 
us with 2',3'-isopropylidene-5'-o-tosyladenosine for 
the preparation of B₃₈-coenzyme. Factor III coenzyme 
(5'-hydroxybenzimidazolyloxylic acid coenzyme) was 
isolated from 2.5 kg of whole cells of *M. omelianskii* 
by the procedures developed by Lezius and Barker 
(5). This B₃₈-coenzyme was crystallized from aqueous 
acetone, where it formed rosettes of orange-colored 
needles (yield, 35 mg). The spectral properties of this 
isolated B₃₈-coenzyme were identical to those reported 
for factor III-coenzyme (5). Ouabain and S-adenosyl-
methionine iodide were obtained from Sigma Chemical 
Co., St. Louis, Mo.

**RESULTS**

When crude extracts of *M. omelianskii* were 
either dialyzed against 0.05 M potassium phos-
phate buffer at pH 7.0 for 2 hr or were passed 
down a Sephadex G 25 column, they lost their 
ability to form CH₄ from methylcobalamin. All 
attempts to activate such extracts by the addition of 
numerous cofactors were unsuccessful. How-
ever, the addition of acetone-fractionated extracts 
(component B) to Sephadex G 25-treated extracts 
(component A) restored significantly methane-
forming ability; however, each component 
showed little activity when tested separately (Fig. 
1).

Optimal stimulation of the rate of CH₄ forma-
tion from methylcobalamin was achieved by the 
addition of ATP, Mg⁺⁺, and FADH₂-generating 
system (Table 1). When the FADH₂-generating 
system was replaced by a reduced flavin mononu-
cleotide (FMNH₂)-generating system, quite 
substantial activation was observed (1.51 
µmole of 
CH₄ in 30 min), but there was greater stimulation 
with the former system. Of the divalent metal ions 
tested, Mn⁺⁺ replaced Mg⁺⁺ to some extent, but 
none of the other ions showed activating ability 
(Table 2).

These data assume considerable importance 
since identical cofactor requirements were or-
ginally shown for the transfer of the methyl group 
from N⁵-methyltetrahydrofolate in the biosyn-
thesis of methionine from homocysteine by B₃₈-
dependent transmethylases from *E. coli* (3). These 
two systems appear even more parallel in light of 
recent evidence that a B₃₈-protein participates in 
the methane-forming reaction (11). However, the 

![Effect of Sephadex-treated extract (A) and 
acetone-fractionated extract (B) on methane 
formation from methylcobalamin.](http://jb.asm.org/)

**FIG. 1.**

**TABLE 1. Requirements for the formation of CH₄ 
from methylcobalamin.**

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>CH₄ formed (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.75</td>
</tr>
<tr>
<td>Component A</td>
<td>0.44</td>
</tr>
<tr>
<td>Component B</td>
<td>0.08</td>
</tr>
<tr>
<td>FADH₂-generating</td>
<td>0.82</td>
</tr>
<tr>
<td>ATP</td>
<td>0.30</td>
</tr>
<tr>
<td>Mg⁺⁺</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*Conditions were the same as for Fig. 1 except 
that Sephadex-treated extract component A con-
tained 16.3 µg of protein, acetone-fractionated 
extract component B contained 9.4 µg of protein, 
and the reaction time was 30 min.*

two systems show some differences in that S-
adenosylmethionine, one of the products of ATP 
utilization, will replace the requirements for ATP 
and Mg⁺⁺ in the methionine reaction. In our 
investigation, S-adenosylmethionine, B₃₈-coenzyme, 
or factor III coenzyme has not replaced ATP 
in the methane reaction; CH₄ formation did not
Table 2. Effect of divalent metal ions on CH₄ formation

<table>
<thead>
<tr>
<th>Metal ion added</th>
<th>CH₄ formed</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>MgSO₄</td>
<td></td>
<td>1.85</td>
</tr>
<tr>
<td>MnCl₂</td>
<td></td>
<td>1.59</td>
</tr>
<tr>
<td>FeSO₄</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>NiCl₂</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>CoCl₂</td>
<td></td>
<td>0.47</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td></td>
<td>0.32</td>
</tr>
</tbody>
</table>

* Reaction mixtures contained: Sephadex-treated extract (component A), 18.55 mg of protein; acetone-fractionated extract (component B), 10.4 mg of protein; ATP, 10.0 µmoles; appropriate inorganic metal ions, 10.0 µmoles; methylcobalamin, 5.0 µmoles; FADH₂-generating system as for Fig. 1; and potassium phosphate buffer at pH 7.0, 350 µmoles. Total liquid volume, 2.5 ml. Reaction time, 30 min.

Fig. 2. Relation of increasing concentrations of ATP to the formation of CH₄. Each reaction mixture contained: crude extract, 37.2 mg of protein; methylcobalamin, 5.0 µmoles; potassium phosphate buffer at pH 7.0, 760 µmoles; and the appropriate concentration of ATP. Total liquid volume, 1.7 ml. Reaction time, 30 min.

Table 3. Effect of various nucleotide triphosphates on CH₄ formation

<table>
<thead>
<tr>
<th>Addition</th>
<th>CH₄ formed</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate</td>
<td></td>
<td>2.70</td>
</tr>
<tr>
<td>Cytidine triphosphate</td>
<td></td>
<td>2.15</td>
</tr>
<tr>
<td>Uridine triphosphate</td>
<td></td>
<td>2.50</td>
</tr>
<tr>
<td>Guanine triphosphate</td>
<td></td>
<td>2.45</td>
</tr>
<tr>
<td>Inosine triphosphate</td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.35</td>
</tr>
</tbody>
</table>

* Reaction mixture contained: crude extract, 41.0 mg of protein; methylcobalamin, 5.0 µmoles; 10.0 µmoles of the appropriate nucleotide triphosphate; and potassium phosphate buffer at pH 7.0, 760 µmoles. Total liquid volume, 1.70 ml. Reaction time, 15 min.

Increasing concentrations of ADP or adenosine monophosphate (AMP) in reaction mixtures containing ATP and ouabain showed considerable reduction in the rates of CH₄ formation (Fig. 4). It appears that the presence of these nucleotide di- or monophosphates, the products of adenosine triphosphatase activity, regulate the rate of CH₄ formation. In the presence of 40 µmoles of ATP and 50 µmoles of ouabain an approximate 1:1 relationship was shown between ATP utilization and CH₄ evolution (Table 4).

Discussion

In the present study of the cofactor requirements for the CH₄ reaction, methylcobalamin has been used because it can be prepared readily in large quantities. Although we can only speculate on the role of ATP in CH₄ formation, we now know that 1 µmole of ATP is required for each
FORMA...J...CH4 FROM METHYLCOBALAMIN

![Graph](image)

**Fig. 3. Effect of increasing concentrations of ATP on the formation of CH4 in the presence of ouabain.** Each reaction mixture contained: crude extract, 38.0 mg of protein; methylcobalamin, 5.0 μmoles; ouabain, 50 μmoles; potassium phosphate buffer at pH 7.0, 760 μmoles; and the appropriate concentration of ATP. Total liquid volume, 2.0 ml. Reaction time, 30 min.

![Graph](image)

**Fig. 4. Effect of increasing concentrations of AMP or ADP on the formation of CH4 in the presence of ATP.** Reaction mixture contained: crude extract, 41.0 mg of protein; methylcobalamin, 5.0 μmoles; ouabain, 50 μmoles; potassium phosphate buffer at pH 7.0, 760 μmoles; ATP, 10.0 μmoles; and the appropriate concentration of ADP or AMP. Total liquid volume, 2.0 ml. Reaction time, 30 min.

Micromole of CH4 evolved, and that ADP and AMP, the products of adenosine triphosphatase activity, regulate CH4 formation. The latter discovery is interesting because we have shown previously that the presence or absence of ATP regulates C3 transfer in the formation of CH4 from N17,N17-methylenetetrahydrofolate in extracts of *M. omelianskii* (10). It appears that nucleotide phosphates can exert their influence at more than one point in C3 transfer in *M. omelianskii*.

We have confirmed the discovery of Lezius and Barker (5) that factor III and factor III coenzyme are the predominant B12 compounds found in whole cells of *M. omelianskii*; however, we find that the majority of our isolated material exists in the form of factor III coenzyme rather than factor III. Since the cells which we used were harvested when they were most active in CH4 formation, we would conclude that coenzyme synthesis is important at this point in the growth cycle. We have proposed that ATP could be involved in providing a 5'-deoxyadenosyl moiety for the upper axial ligand of a protein-bound reduced factor III complex, and that this 5'-deoxyadenosyl ligand could be replaced by a methyl group from the methyl-donating substrate (7). This proposed reaction mechanism would involve the utilization of one ATP per methyl group transferred. However, the possibility that ATP may be required to generate reducing potential cannot be overlooked.

**Acknowledgments**

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**Literature Cited**


