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

J. M. WOOD AND R. S. WOLFE

Department of Microbiology, University of Illinois, Urbana, Illinois

Received for publication 2 May 1966

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-cobalt-5,6-dimethylbenzimidazolycobamide) 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$_1$ 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 Stadtman (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 thermoaceticum (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 μmole of
nicotinamide adenine dinucleotide (NAD), 0.04 μmoles of flavin adenine dinucleotide (FAD), and 40.0 μmoles 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). B₂₄-coenzyme (5,6-dimethylbenzimidazolylcobamide) was synthesized 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 B₂₄-coenzyme. Factor III coenzyme (5'-hydroxybenzimidazolylcobamide) was isolated from 2.5 kg of whole cells of M. omelianskii by the procedures developed by Lezius and Barker (5). This B₂₄-coenzyme was crystallized from aqueous acetone, where it formed rosettes of orange-colored needles (yield, 35 mg). The spectral properties of this isolated B₂₄-coenzyme were identical to those reported for factor III-coenzyme (5). Ouabain and S-adenosylmethionine 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 phosphate 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. However, 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 formation from methylcobalamin was achieved by the addition of ATP, Mg²⁺, and FADH₂-generating system (Table 1). When the FADH₂-generating system was replaced by a reduced flavin mononucleotide (FMNH₂)-generating system, quite substantial activation was observed (1.51 μmoles of CH₄ in 30 min), but there was greater stimulation with the former system. Of the divalent metal ions tested, Mn²⁺ replaced Mg²⁺ to some extent, but none of the other ions showed activating ability (Table 2).

These data assume considerable importance since identical cofactor requirements were originally shown for the transfer of the methyl group from N⁵-methyltetrahydrofolate in the biosynthesis of methionine from homocysteine by B₂₄-dependent transmethylation in E. coli (3). These two systems appear even more parallel in light of recent evidence that a B₂₄-protein participates in the methane-forming reaction (11). However, the two systems show some differences in that S-adenosylmethionine, one of the products of ATP utilization, will replace the requirements for ATP and Mg²⁺ in the methionine reaction. In our investigation, S-adenosylmethionine, B₂₄-coenzyme, or factor III coenzyme has not replaced ATP in the methane reaction; CH₄ formation did not
evolved in reaction mixtures —trahs also removed pH of jumole iodide was —treated {CoCl2.. {CoCl2.. 0.47

The above...

mation, tions of ATP used... ouabain in inclusion...

APPENDIX 2. Effect of divalent metal ions on CH4 formation

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

exceed that of control reaction flasks when 10.0 μmoles of B2-coenzyme, factor III coenzyme, or S-adenosylmethionine iodide was added to reaction mixtures containing 32.4 mg of crude extracts and 5.0 μmoles of methylcobalamin. When ATP was substituted for the above compounds in the reaction mixture, 4.0 μmoles of CH4 was evolved in 20 min. When 5.0 μmoles of methylcobalamin was allowed to react in the presence of increasing concentrations of ATP with crude extracts of M. omelianskii, it was observed repeatedly that the number of micromoles of ATP required to form 1 μmole of CH4 was greater than 1.0 (values varied between 2.0 and 1.6). Concentrations of ATP above the approximate 2:1 ratio showed a marked inhibition in the rate of CH4 formation (Fig. 2).

It was observed by M. J. Wolin that reaction mixtures in which methylcobalamin had been omitted also removed ATP at a rapid rate; this indicated the presence of a powerful adenosine triphosphatase in the extracts. Adenosine diphosphate (ADP) previously was shown to stimulate CH4 formation but to a lesser extent that ATP (9). Other nucleotide triphosphates, with the exception of inosine triphosphate, were equally efficient in stimulating this reaction (Table 3).

The inhibition of the methane reaction by high concentrations of ATP was explained through the use of ouabain as inhibitor for adenosine triphosphatase. The inclusion of 50 μmoles of ouabain in reaction mixtures caused greater than 96% inhibition of adenosine triphosphatase. In the presence of this inhibitor, increasing concentrations of ATP did not inhibit the rate of CH4 formation, but the reaction assumed normal saturation kinetics (Fig. 3). However, inclusion of 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).

**Table 3. Effect of various nucleotide triphosphates on CH4 formation**

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

* 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.
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 C₄ transfer in the formation of CH₄ from N⁷,N¹⁰-methylene-tetrahydrofolate in extracts of M. omelianskii (10). It appears that nucleotide phosphates can exert their influence at more than one point in C₄ 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

We wish to thank Norman Ryckman, Jack Althaus, and Sally Forbes for technical assistance. We acknowledge the interest of our colleagues E. A. Wolin and M. J. Wolin.

This investigation was supported by National Science Foundation grant GB-1878 and by Public Health Service grants WP-0016 and E-4445.

LITERATURE CITED


