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Formation of Methane from Methyl Factor B and Methyl Factor III by Cell-Free Extracts of *Methanobacillus omelianskii**

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ABSTRACT: Methylcobinamide (methyl factor B) and methyl-Co-5-hydroxybenzimidazolylcobamide (methyl factor III) were tested as methyl donors for the transmethylation reaction leading to the formation of methane in extracts of the methane bacterium *Methanobacillus omelianskii*. The methyl-cobalt ligands of both substrates were approximately as active as that of methyl-Co-5,6-dimethylbenzimidazolylcobamide (methylcobalamin); the presence of the dimethylbenzimidazole nucleotide moiety is not required for the methyl–cobalt ligand to be active as methyl donor Adenosine triphosphate was required for the formation of methane from both methyl-B_{12} analogs. The properties of derivatives prepared from the respective reduced cobinamide and cobamide products which accumulate suggest that they possess sulfhydryl radicals on their respective cobalt atoms. The striking lack of specificity of methyl-B_{12} analogs as methyl donors in biological systems is postulated to reside in their ability to chemically alkylate reduced corrinoid-containing enzymes.

To test the specificity of the enzyme system in *Methanobacillus omelianskii* which converts methylcobalamin[1] to methane (Wolin *et al.*, 1963, 1964), methyl factor B (Müller and Müller, 1962) and methyl factor III were chosen as substrates. Methyl factor B may be regarded as methylcobalamin with the exclusion of the benzimidazole nucleotide moiety, and factor III derivatives have been shown to be the predominant, naturally occurring cobamide compounds present in *M. omelianskii* (Lezius and Barker, 1965).

Experimental Section

Factors B and III were generous gifts from Dr. L. Mervyn of Glaxo Research Limited, Greenford, Middlesex, England. Methyl factor B was synthesized by methylating hydrido factor B with dimethyl sulfate at 0°, and its absorption spectrum was compared with a sample of methyl factor B kindly provided by Dr. D. H. Dolphin. Spectra were determined by use of a Cary Model 14 spectrophotometer. Hydrido factor B was

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[1] Abbreviations used in this work: methyl factor B, methylcobinamide; methyl factor III, methyl-Co-5-hydroxybenzimidazolylcobamide; methylcobalamin, methyl-Co-5,6-dimethylbenzimidazolylcobamide; ATP, adenosine 5′-triphosphate.

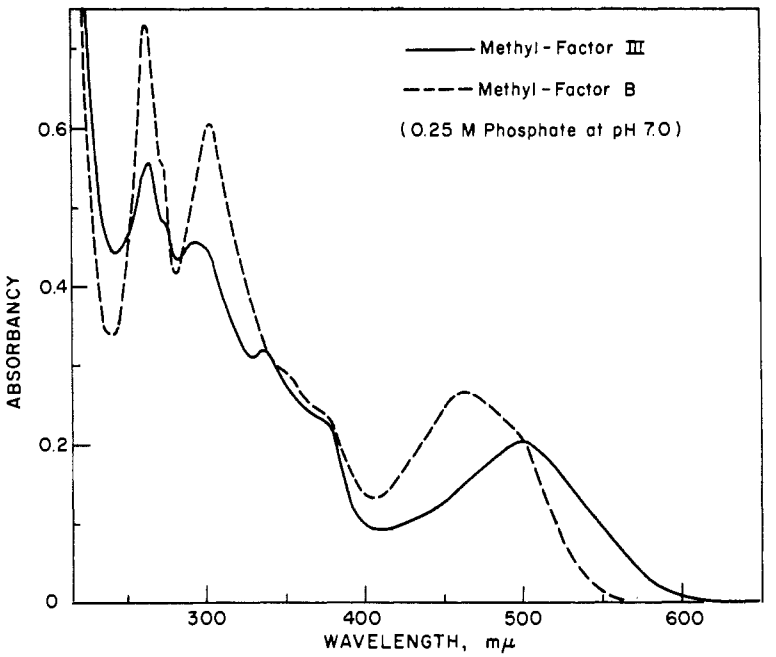

FIGURE 1: Absorption spectra of methyl factor B (- - - -) and methyl factor III (———) in 0.25 M potassium phosphate buffer, pH 7.0.

prepared by the borohydride reduction method outlined by Johnson *et al.* (1963). [^{14}C]Methyl factor III (sp act. 47.2 mc/mmole) was synthesized by treating 0.5 mc of [^{14}C]methyl iodide (sp act. 56.25 mc/mmole) with hydrido factor III.

Cells of *M. omelianskii* were mass cultured, harvested, and washed as described by Johns and Barker (1960) with modifications by Wolin *et al.* (1963). Cell-free extracts were prepared by exposing 1 g of cells (wet weight)/ml of 0.5 M potassium phosphate buffer, pH 7.0, to the maximum frequency output of a Branson sonic probe for 2 min at 0°. Cell debris was removed by centrifugation at 23,000*g* for 20 min at 0°. In all reactions extracts were used immediately after their preparation, and the protein concentration of each extract was determined by the biuret procedure outlined by Gornall *et al.* (1949). Methane and [^{14}C]-methane were assayed by the method described by Wood *et al.* (1965) and Wood and Wolfe (1965).

Results

A comparison of the absorption spectra of the two substrates, methyl factor III and methyl factor B, is presented in Figure 1. The absorption spectrum of methyl factor B has been documented previously (Müller and Müller, 1962).

Crude extracts of *M. omelianskii* catalyzed the stoichiometric evolution of methane from methyl factor B and methyl factor III only in the presence of ATP (Figure 2). A comparison of the rates of methane formation from methyl factor B and methylcobalamin under the conditions outlined for Figure 2b indicates that methylcobalamin is a better substrate for the methane-forming enzyme; in identical reaction mixtures 3.0 μmoles of methane was formed in 10 min from methylcobalamin compared with 1.2 μmoles when methyl factor B was the substrate.

The results of the following experiments lead us to believe that the reduced cobinamide and/or cobamide products which accumulate after methane formation has ceased contain sulfhydryl radicals on their respective cobalt atoms. When 50.0 μmoles of methyl iodide was injected into a reaction flask which contained the reduced cobinamide product, or into a flask which contained the reduced cobamide product, immediate reactions at 0° yielded an orange cobinamide and a red cobamide product. These products, the result of chemical alkylation, were identified as methyl factor B and methyl factor III after spectrophotometric and chromatographic analyses.

In additional experiments, reaction flasks which contained the reduced cobinamide or cobamide products were shaken in air, and the resulting oxidized B_{12} compounds were extracted by the method outlined by Johnson *et al.* (1963). The absorption spectrum of the oxidized cobinamide product was identical with that reported for authentic hydroxy factor B (Toohey, 1965); the absorption spectrum of the oxidized cobamide product was similar to that of chemically synthesized hydroxy factor III (aquo factor III, Figure 3).

When both of these oxidized products were incubated with alkaline KCN, dicyano factor B and cyano factor III were identified as the products of cyanolysis (Fried-

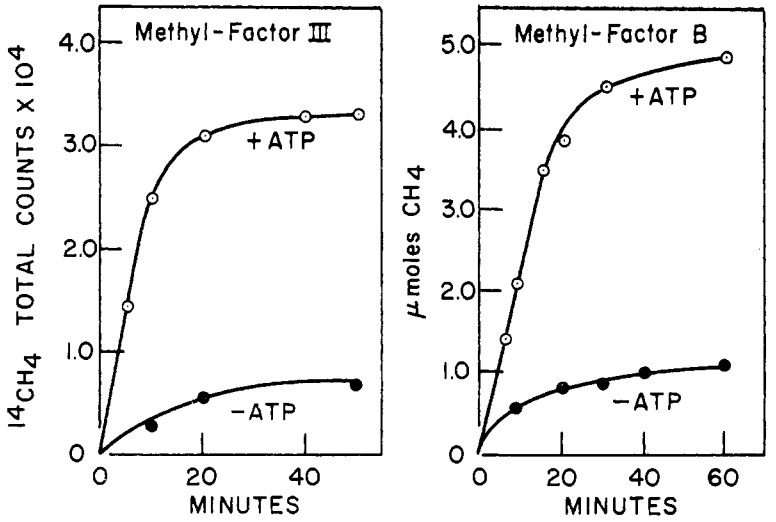

FIGURE 2: Formation of [^{14}C]methane from [^{14}C]methyl factor III (a) and formation of methane from methyl factor B (b). (a) The reaction mixtures contained: crude extract, 20.05 mg of protein; [^{14}C]methyl factor III (sp act. 47.2 mc/mmole), 0.73 μmole; ATP (where indicated), 5.0 μmoles. Total reaction volume 0.75 ml at 40° under H_2. (b) The reaction mixtures contained: crude extract, 87.0 mg of protein; methyl factor B, 4.0 μmoles; potassium phosphate buffer, pH 7.0, 760 μmoles; ATP (where indicated), 10.0 μmoles. Total reaction volume 2.0 ml at 40° under H_2.

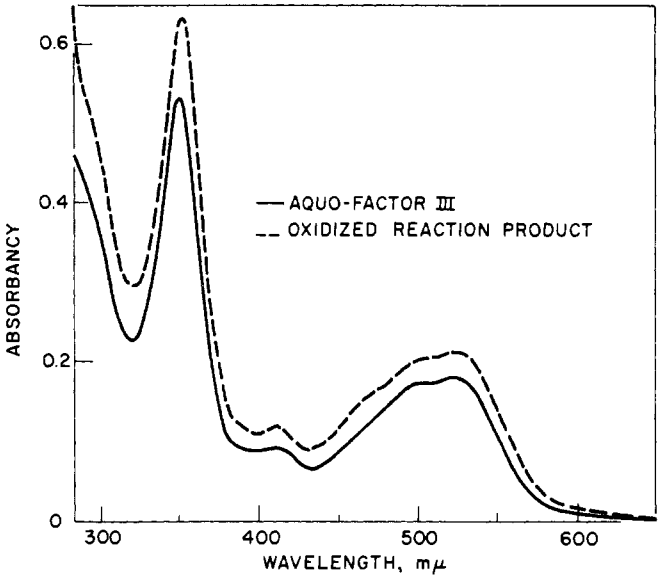

FIGURE 3: Comparison of the spectrum of the oxidized product of enzymic demethylation of methyl factor III (- - - -), and the spectrum of aquo factor III (hydroxy factor III) (———). Both spectra were recorded in 0.25 M potassium phosphate buffer at pH 7.0.

rich and Bernhauer, 1956). The appearance of the strong absorption band at 368 mμ, after cyanolysis, indicates that the corrin chromophore has not been altered during the demethylation of both methyl-B_{12} analogs.

Discussion

Although N^5-methyltetrahydrofolate has been established as an intermediate in methane formation from C-3 of serine in cell-free extracts of *M. omelianskii* (Wood *et al.*, 1965) no evidence has been found to support the existence of a free cobamide or cobinamide compound as the ultimate methyl donor in this metabolic pathway, even though methylcobalamin, methyl factor B, and methyl factor III have been shown to be excellent substrates for the methane-forming enzyme. The methyl–cobalt ligand could serve as a gratuitous methyl donor for the methane-forming enzyme system, or it could be metabolized by a pathway independent of the tetrahydrofolate sequence.

The naturally occurring cobamide compounds in

M. omelianskii have been isolated recently by Lezius and Barker (1965), and have been shown to be predominantly factor III (5-hydroxybenzimidazolylcobamide) and factor III coenzyme (Co-5′-deoxyadenosyl derivative of factor III). Although methyl factor III functions as a methyl donor in extracts of *M. omelianskii*, the role of this compound as a free intermediate in methane formation is questionable. Our inability to demonstrate the existence of a free corrinoid compound as an intermediate in methane formation parallels the results reported for the transfer of the methyl group from N^5-methyltetrahydrofolate in the biosynthesis of methionine from homocysteine in *E. coli* by the enzyme, 5-methyltetrahydrofolate homocysteine transmethylase. Weissbach *et al.* and Brot and Weissbach (1965) have shown that this enzyme contains a bound cobamide compound which may be the active site for methyl transfer. We have presented evidence recently that a similar B_{12} protein is involved in the methyl-transfer reaction leading to the formation of methane (Wood and Wolfe, 1966). Since methyl factor B also will substitute for methylcobalamin as methyl donor in the biosynthesis of methionine in *E. coli* (Elford *et al.*, 1965), and since methylcobyric acid, methylcobalamin, and methyl factor IIIm (methyl-Co-5-methoxybenzimidazolylcobamide) will all serve as methyl donors in the methyltransfer reaction leading to the biosynthesis of the methyl group of acetate in extracts of *Clostridium thermoaceticum* (Ljungdahl *et al.*, 1965), the possibility of chemical alkylation of reduced B_{12} proteins by a variety of methyl-B_{12} analogs in all these systems appears likely (Wolfe *et al.*, 1966). The ease with which B_{12} proteins can be alkylated with reagents such as propyl iodide favors this hypothesis.

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