REDUCTION AND ALKYLATION OF THE COBAMIDE PROSTHETIC GROUP IN THE ENZYMATIC SYNTHESIS OF METHIONINE

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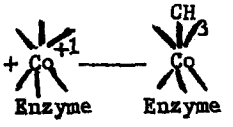
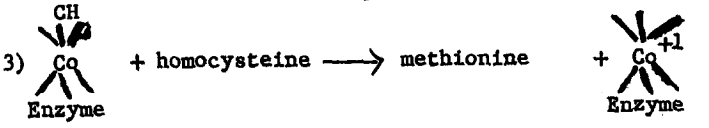
It has been postulated from recent studies that a reduced cobamide functions in the terminal step (Reaction 1) in the biosynthesis of methionine (4,5,8,9).

1) N^5-Methyl-folate-H_4 + homocysteine $\xrightarrow[\text{reducing system}]{\text{S-adenosylmethionine } B_{12}\text{ protein}}$ methionine + folate-H_4

The methyl transfer is most easily pictured as a methyl carbonium ion transfer from N^5-methyl-folate-H_4 to a reduced cobamide on the enzyme forming a transient methyl-B_{12}[2] enzyme (4,5,8). Subsequent transfer of the methyl group to homocysteine regenerates the reduced cobamide bound to the enzyme (Reactions 2 and 3).

2) N^5-Methyl-folate-H_4 +  + folate-H_4

3)  + homocysteine ⟶ methionine +

Studies on the in vitro formation of holoenzyme (reaction of apoenzyme with a cobamide) have emphasized the importance of the carbon-to-cobalt bond in methionine synthesis (11). Incubation of apoenzyme with propyl-B_{12} formed an inhibited enzyme, which upon exposure to light yielded an active holoenzyme. The known light sensitivity of the carbon-to-cobalt bond (3,7) suggested the following sequence of reactions (11):

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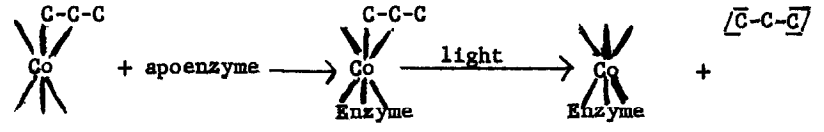
2. The following abbreviation will be used: methyl-B_{12}, 5,6-dimethyl-benzimidazolylcobamide-methyl. The other cobamide derivatives are abbreviated in the same way.



Propyl-B_{12} Inhibited enzyme (propyl-B_{12} enzyme) (holoenzyme)

The valence state of the cobamide present in holoenzyme is not known, and although reduction of the cobalt is felt to be an essential step in the over-all reaction, direct proof that a reduced cobamide can be formed on the enzyme, and functions in methyl transfer has not yet been obtained. The known chemical reactivity of hydrido-B_{12} (B_{12s}), a reduced cobamide containing monovalent cobalt (6), and cobamide derivatives formed by reduction with sodium hydrosulfite (2), suggested a possible approach to this problem. These cobamide derivatives rapidly react with alkyl halides to form alkyl cobamides (1,2,6). A similar alkylation reaction with an alkyl halide conceivably could occur on the enzyme if the bound cobamide is reduced during the enzymatic reaction. If the alkylating agent employed is propyl iodide a propyl-B_{12} enzyme would be formed, which although inactive, could be determined by its conversion to an active enzyme by visible light (11). In the present report evidence is presented that N^5-methyl-folate-H_4 homocysteine methyl transferase holoenzyme, when incubated in the presence of a reduced pyridine nucleotide, FAD, S-adenosylmethionine, and propyl iodide forms an inactivated enzyme. Exposure to light reactivates the enzyme which indicates that alkylation of the protein-bound cobamide has occurred.

Escherichia coli K_{12} (strain 2276, a methionine-cyano-B_{12} auxotroph) was obtained from Dr. A. L. Taylor of the National Institutes of Health. The organism was grown in a glucose minimal salt medium containing 0.1 μg of vitamin B_{12} (cyano-B_{12}) per ml. The methyl-folate-H_4-homocysteine methyl transferase obtained from these cells was entirely in the form of holoenzyme. A 25 - 45 percent ammonium sulfate fraction was prepared and dialyzed as described previously (9). This fraction was then passed through a Sephadex G-25 column, and the protein eluate used for the experiments reported here.

Preincubations contained in a total volume of 0.2 ml, 0.1 ml of Sephadex treated holoenzyme (1.3 mg protein), 10 μmoles potassium phosphate buffer pH 7.4, 1 μmole TPNH, 2 mμmoles FAD, 2 μmoles propyl iodide (in 0.01 ml ethanol), and 10 mμmoles of S-adenosylmethionine. After incubation at 37° C for 15 minutes 1.4 ml of cold water were added and the reaction tubes placed in ice and kept dark. Aliquots of this reaction mixture (0.04 ml) were exposed in the cold to a 200-watt tungsten lamp for 5 minutes at a distance of 15 cm, and then assayed for their ability to catalyze methyl transfer from N^5-methyl-^{14}C-folate-H_4 to homocysteine as previously reported (8,10). The activity of the light exposed sample was compared to the activity of a similar sample from the preincubation mixture which had been kept dark. Since a propyl-B_{12} enzyme is inactive in catalyzing methionine formation from N^5-methyl-folate-H_4, but can be activated by light (11), an increase in the enzymatic activity after illumination was used as a measure of the presence of a propyl-B_{12} enzyme. A typical experiment is shown in Table I. Incubation of the cobamide holoenzyme with propyl iodide and the other components present in the complete system, resulted in a marked inhibition of the enzyme activity, which could be reversed by exposure of the preincubation mixture to visible light. As shown in Table I this effect was dependent on TPNH, S-adenosylmethionine, FAD and propyl iodide and did not occur if the preincubation was done at 0°. TPN could not replace TPNH. Butyl iodide, which should also yield a light sensitive inhibited enzyme (11,12), gave similar results as propyl iodide.

Methyl iodide was also tested in this system as shown in Table I. This alkyl halide would be expected to yield a methyl-B_{12} enzyme during the preincubation. However, unlike the propyl-B_{12} or butyl-B_{12} enzymes, a methyl-B_{12} enzyme is visualized as an intermediate in methyl transfer from N^5-methyl-folate-H_4 to homocysteine (Reactions 2 and 3), and should not be activated by visible light. The results in Table I support this view. In a series of experiments in which propyl iodide was replaced by methyl iodide no significant formation (0 - 10%) of a light sensitive inhibited enzyme was obtained.

Table 1

REQUIREMENTS FOR ALKYLATION OF HOLOENZYME

Preincubations[1]	mμMoles methionine formed/30' Dark	Light	% Inhibition[3]
Expt. I			
Complete system	1.4	3.0	53
- TPNH	2.9	2.8	0
- FAD	2.6	2.5	0
- S-adenosylmethionine	2.7	2.6	0
- Propyl iodide[2]	3.7	3.6	0
- Propyl iodide + butyl iodide	1.4	2.9	52
- Propyl iodide + methyl iodide	2.5	2.5	0
Complete system 0°	3.3	3.2	0
Expt. II			
Complete system	1.5	2.8	46
+ N^5-methyl-folate-H_4[4]	2.3	2.6	11
+ N^5-propyl-folate-H_4	1.3	2.6	50
- Propyl iodide + N^5-propyl-folate-H_4	2.5	2.6	<5

[1] The components in the preincubation mixture, and the conditions used are described in the text. Butyl- and methyl iodide were used at the same concentration as propyl iodide (2 μMoles).

[2] Ethanol which was used to dissolve the propyl iodide was substituted for the propyl iodide in this sample.

[3] Inhibition of the enzyme activity of the extracts kept in the dark compared to those exposed to light. With longer periods of preincubation it was possible to obtain as high as 90% inhibition.

[4] Thirty mμmoles of the folate derivatives (DL) were added where indicated. N^5-propyl-folate-H_4 was kindly supplied by Dr. J. Keresztesy of the National Institute of Arthritis and Metabolic Diseases, NIH.

As shown in Experiment II, Table 1, 30 mμmoles of N^5-methyl-folate-H_4, the natural methyl donor in the enzymatic reaction, prevented the alkylation of the enzyme by propyl iodide. N^5-propyl-folate-H_4 did not have this effect. It should also be noted that N^5-propyl-folate-H_4 did not form a propyl-B_{12} enzyme when used in place of propyl iodide.

The experiments described above indicate that the cobamide prosthetic group, present in the N^5-methyl-folate-H_4-homocysteine methyltransferase holoenzyme, can be converted to a form capable of reacting with an alkyl halide.

The need for a reducing system, in conjunction with the known chemical reactivity of reduced cobamides (1,2,6) favors the view that reduction of the enzyme-bound cobamide is a necessary feature of the alkylation reaction. The alkylation of the enzyme can be pictured as a partial reaction (Reaction 2) in the over-all reaction.

The requirements for this reaction (S-adenosylmethionine, and a reducing system) are the same as the requirements for the enzymatic transfer of the methyl group from N^5-methyl-folate-H_4 to homocysteine (Reaction 1). In the enzymatic reaction N^5-methyl-folate-H_4 is pictured as the alkyl donor, analagous to the reaction described here with propyl iodide.

Since N^5-methyl-folate-H_4 prevented alkylation of the enzyme by propyl iodide, one would expect that any compound that is able to methylate the cobamide on the enzyme would inhibit the reaction of propyl iodide with holoenzyme. The failure of S-adenosylmethionine to inhibit the formation of a propyl-B_{12} enzyme from propyl iodide (S-adenosylmethionine is actually required for the reaction) suggests that S-adenosylmethionine does not transfer its methyl group to the holoenzyme to form a methyl-B_{12} enzyme. The possibilities should be considered, from the above data, that S-adenosylmethionine functions in the reaction by methylation of a noncobamide site, and/or as an allosteric agent.

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