SPECTROPHOTOMETRIC EVIDENCE FOR THE FORMATION OF AN *ESCHERICHIA COLI* B B_{12s} METHYLTRANSFERASE[1]

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Summary. Upon the addition of AMe to a 10 μM solution of B_{12} enzyme in a FH_4 + DTT reducing system containing homocysteine, the absorption spectrum of the cobalamin chromophore changed from a B_{12r} type of spectrum (470-475 mμ peak) to a B_{12s} spectrum (maxima at 385 mμ and 460 mμ). These spectral changes began immediately after the injection of AMe at 37° and became maximal 6-12 minutes later. The original B_{12r} spectrum was restored after approximately 24 minutes but was converted back to a B_{12s} spectrum upon a second addition of exogenous AMe. In a parallel experiment the transient appearance of the B_{12s}-like absorption spectrum was correlated with the metabolism of methyl-^{14}C-AMe, i.e., the transfer of methyl-^{14}C groups to homocysteine. Steady-state catalysis of N^5-methyl-^{14}C-FH_4-homocysteine transmethylation in the FH_4 + DTT reducing system was dependent on exogenous AMe and a non-enzymatic reduction of free hydroxy-B_{12} to B_{12s} was not observed in this reducing system.

E. coli B cobalamin methyltransferase catalyzes Reaction 1

1) N^5-Methyl-FH_4 + homocysteine $\xrightarrow[FMNH_2,\ DTT,\ AMe]{B_{12}\ enzyme}$ FH_4 + methionine

optimally in a $FMNH_2$ + DTT reducing system containing AMe (1). As a result of a series of experiments carried out with this reducing system and purified B_{12} enzyme, a schematic mechanism for Reaction 1 was proposed (2) in which AMe was assigned a pre-methylation function and a Co^{+1} enzyme (B_{12s} enzyme) was

[1] Abbreviations: cyano-B_{12}, cyanocobalamin (5,6-dimethylbenzmidazolyl-cyanocobamide; hydroxy-B_{12}, hydroxo (aquo) cobalamin; B_{12s}, a 2-electron reduced form of hydroxy-B_{12} containing Co^{+1}; B_{12r}, a 1-electron reduced form of hydroxy-B_{12} containing Co^{+2}; methyl-B_{12}, methyl-cobalamin; N^5-methyl-FH_4, *dl*, N^5-methyltetrahydrofolate; B_{12} enzyme, non-methylated N^5-methyl-FH_4-homocysteine cobalamin methyltransferase; AMe, S-adenosyl-L-methionine iodide, DTT, 1,4-dithiothreitol.

depicted as the methyl group acceptor for N^5-methyl-FH_4 (see Fig. 11 in Ref. 2). A B_{12s} enzyme would be regenerated upon the subsequent transfer of the methyl group to homocysteine as a carbonium ion. Until now, the existence of a labile B_{12s} enzyme complex has only been inferred from the extremely short catalytic life of an AMe (or methyl iodide) pre-methylated B_{12} enzyme (2), and more recently a reconstituted methyl-^{14}C-B_{12} enzyme (3), when only the two substrates were added. Moreover, it was not possible to detect the formation of a B_{12s} enzyme spectrophotometrically in the presence of $FMNH_2$ because of the intense absorption given by traces of oxidized flavin in the 380 mμ and 450 mμ regions. To circumvent this problem we used 3.0 mM FH_4 as a reducing agent. Although it is the co-product in Reaction 1, it has been shown to satisfy partially the requirement for $FMNH_2$ at concentrations of 0.5-1.5 mM (2).

Methods and Materials. Most of the pertinent methodology as well as the sources of most of the materials have been adequately described (1-5). All radioactivity measurements were taken with a Packard Tri-Carb liquid scintillation spectrometer with the use of a water miscible counting fluid (6). Absorption spectra were taken with a Cary recording spectrophotometer equipped with a 0-0.2 absorbance expanded scale. The B_{12} enzyme employed here was the same preparation that was used in References 3 and 4. It was prepared by a slight modification (4) of a published procedure (1) and contained 1.0 mμmole of bound cobalamin/mg of protein (1, 7). FH_4 was purchased from the Sigma Chemical Co.

Results and Discussion. According to the reaction scheme in Figure 11 of Reference2, the initially inactive B_{12} enzyme that is customarily purified (1, 4) should not yield a functional Co^{+1} (B_{12s}) chromophore until it has been methylated by AMe and then de-methylated. In order to observe the maximal

concentration of B_{12s} enzyme for a period of time, excess exogenous AMe would be essential. Excess homocysteine rapidly de-methylates a methyl-B_{12} enzyme (2) and thus would prevent the accumulation (2, 5) of N^5-methyl-FH_4. Figure 1

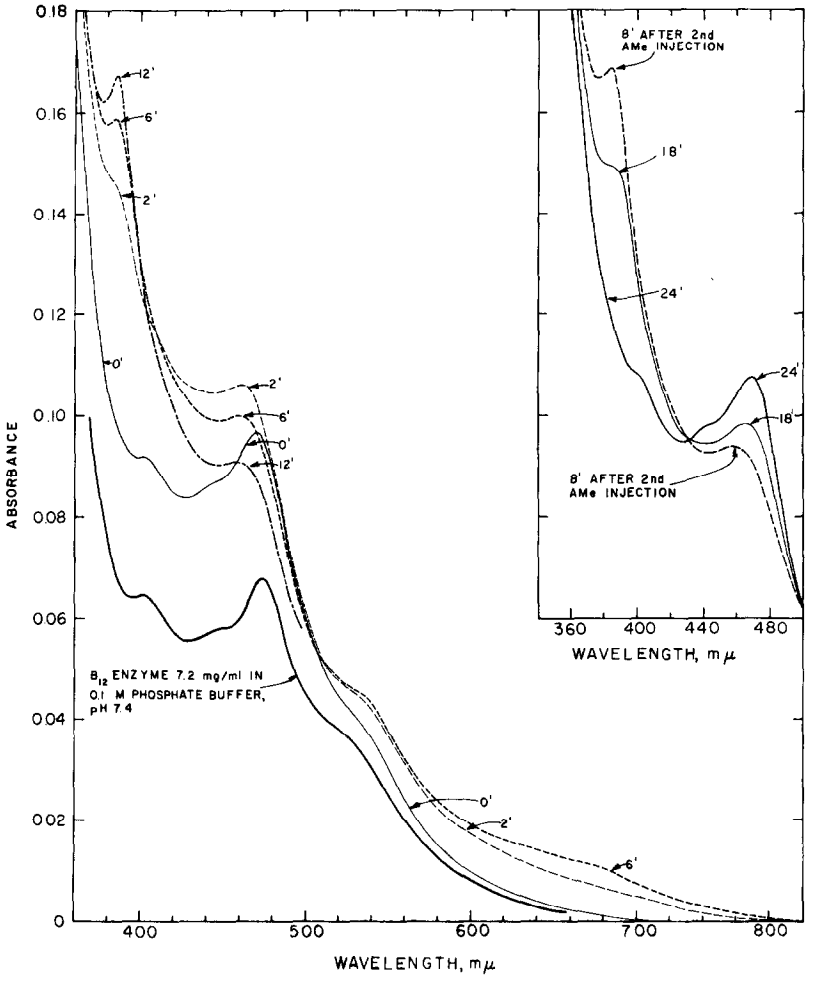

Fig. 1. AMe dependent formation of a B_{12s} enzyme. Complete cuvette mixture (0.98 ml) contained potassium phosphate buffer, 0.1 M, pH 7.4; FH_4, 3.0 mM; DTT, 25 mM; homocysteine 1.0 mM; and B_{12} enzyme, 10 μM. Reference cuvette mixture (0.98 ml) was identical except that water was substituted for the B_{12} enzyme. Each cuvette was fitted with a rubber serum cap and the air phase was replaced with H_2 by gassing (1) for 15 min at 22° with agitation. Both cuvette mixtures were then equilibrated for 5 min at 37° and an absorption spectrum (0 min) was taken. AMe, 20 μl of a pre-H_2 gassed 10 mM solution, was then injected into each cuvette and spectra were recorded as indicated during the subsequent 24 min. At the end of 24 min, 20 μl of AMe were again injected into each cuvette and one more spectrum is depicted (inset) 8 min later. Throughout the experiment the temperature was maintained as close as possible to 37°.

shows that an incubation in the presence of FH_4, DTT, and homocysteine did not alter the B_{12r}-like absorption spectrum given initially by the B_{12} enzyme in phosphate buffer. Immediately upon the injection of AMe, however, the absorption spectrum of the bound cobalamin was observed to change. Within 2 minutes the 470 mμ peak began to shift to a lower wavelength, a large shoulder appeared at 380-390 mμ, and there were small increases in the 545 mμ and 700 mμ regions. These changes became maximal 6-12 minutes after the addition of AMe and resulted in distinct maxima at 385 mμ and 460 mμ. The 385 mμ peak began to decrease after 12 minutes as the 460 mμ peak simultaneously shifted back to 470 mμ and intensified. After 24 minutes the original B_{12r}-like absorption spectrum of the enzyme had substantially reformed (Fig. 1, inset). Upon a second addition of AMe the 385 mμ and the 460 mμ absorption maxima again formed (Fig. 1, inset, 8 min) and then disappeared (not shown) reversibly. Virtually the same transient AMe dependent spectral changes seen in Figure 1 were obtained with 10 mg of methyl-^{14}C-B_{12} enzyme which had been methylated with methyl-^{14}C-AMe and then re-isolated.

Figure 2 shows that free hydroxy-B_{12} was not reduced non-enzymatically beyond the B_{12r} or Co^{+2} stage under the exact same incubation conditions that were used in Figure 1. Also, it may be noted in Figure 2 that B_{12s}, which was prepared by reduction with $NaBH_4$, displayed an intense absorption peak at 385 mμ as well as less prominent maxima at 460 mμ, 545 mμ, and perhaps 680 mμ. The spectrum of B_{12s} in Figure 2 agrees with published spectra (8, 9) for this extremely labile compound which has only been detected in solutions. A prominent absorption peak at 385 mμ coupled with a much smaller 460 mμ peak is unique for B_{12s} among the reported cobalamins (10). These two absorption maxima in Figure 2 correspond to the peaks given by the B_{12} enzyme in Figure 1 upon the addition of AMe.

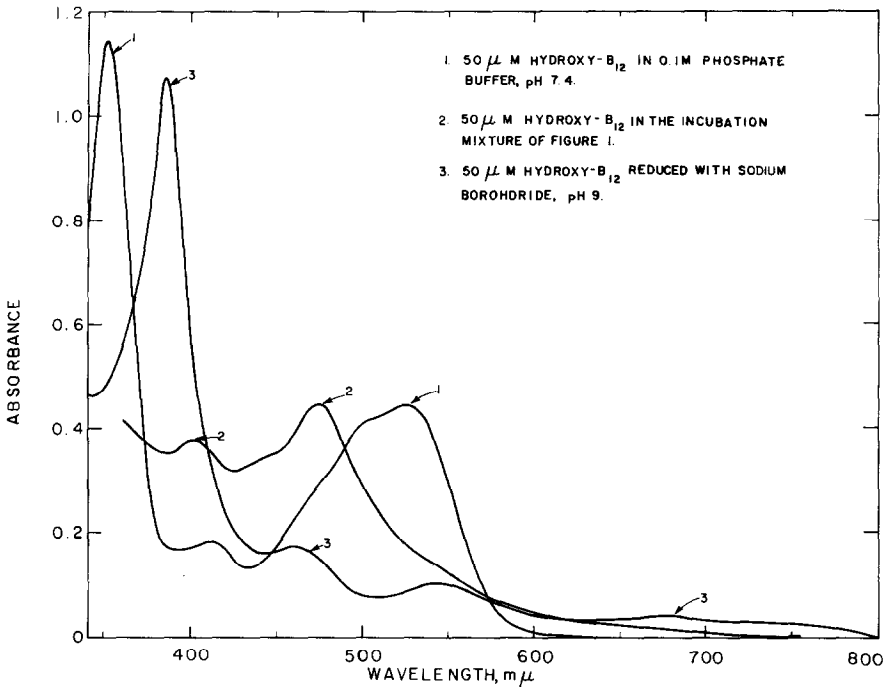

Fig. 2. Effect of the incubation system in Fig. 1 on free hydroxy-B_{12}. Curve 1, hydroxy-B_{12}. Curve 2, hydroxy-B_{12} before and up to 20 min after the injection of AMe at 37°; experimental procedure and conditions for curve 2 were the same as in Fig. 1. Curve 3, 2 hours after the addition of 2 mg of $NaBH_4$ to 1.0 ml of 50 μM hydroxy-B_{12} in water (H_2 gas phase).

Figure 3A shows, with methyl-^{14}C-AMe, that under the incubation conditions in Figure 1 most of the AMe was transmethylated to homocysteine at the end of 18 minutes. Methyl-^{14}C-AMe-homocysteine transmethylation is catalyzed by the B_{12} enzyme at < 1% of the rate of N^5-methyl-^{14}C-FH_4-homocysteine transmethylation (1, 2, 11); however, with the use of 10 mμmoles of B_{12} enzyme in Figure 1 this rate was sufficient to metabolize most of the added AMe, 0.2 μmole, in about 20 minutes. Our failure to show the complete metabolism of 20 mμmoles of methyl-^{14}C-AMe to methyl-^{14}C-methionine in Figure 3 was due, in part, to the instantaneous but nonspecific, very tight binding of 2.5 mμmoles of methyl-^{14}C-AMe to protein in the B_{12} enzyme prepara

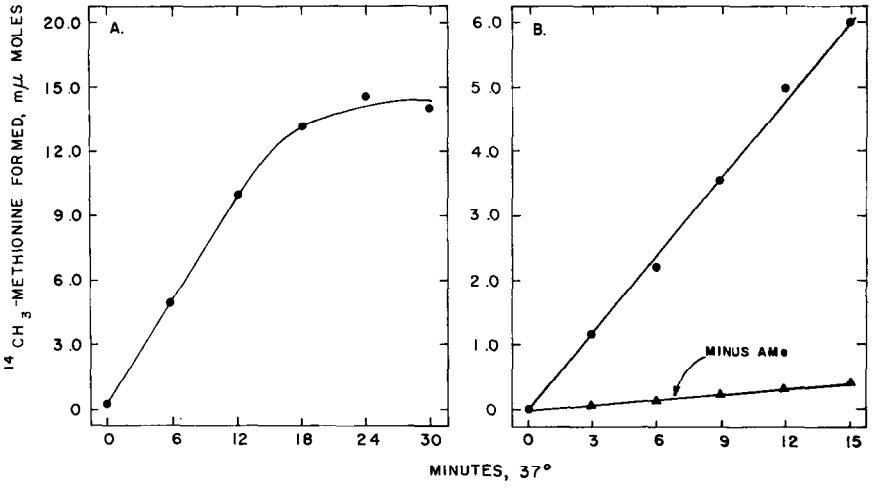

Fig. 3. Catalysis of methyl-^{14}C-AMe-homocysteine (A) and N^5-methyl-^{14}C-FH_4-homocysteine (B) transmethylation in the FH_4 + DTT reducing system of Fig. 1. (A) Incubation systems were identical to the complete system in Fig. 1 except that they were proportionately scaled down 10-fold to a final volume of 0.1 ml and they contained methyl-^{14}C-AMe (4200 cpm/mμmole), 20 mμmoles. Complete systems, including the methyl-^{14}C-AMe, were pre-gassed 5 min at 0° in 10 x 75 mM tubes (1) and then incubated at 37° under H_2. Reactions were terminated with 0.9 ml of ice-cold water and methyl-^{14}C-methionine was determined with the use of Bio-Rex-70-acetate plus Dowex-1-Cl^- columns (2). (B) Complete reaction mixtures (0.2 ml) contained the same concentrations of phosphate buffer, FH_4, DTT, homocysteine, and unlabeled AMe as in Fig. 1 and in addition they contained N^5-methyl-^{14}C-FH_4 (2000 cpm/mμmole), 60 mμmoles. The amount of B_{12} enzyme was decreased however to 1.3 μg. These systems were pre-gassed with H_2 for 5 min at 0° (1) and then incubated for 15 min at 37°. Catalysis was terminated with 0.8 ml of ice-cold water and methyl-^{14}C-methionine formation was determined with the use of Dowex-1-Cl^- columns (1).

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tion. This irreversible binding has been described previously (5). It occurs at 0° and the bound methyl-^{14}C-AMe then behaves like radioactive methionine on Bio-Rex-70-acetate columns. Therefore 2.5 mμmoles was subtracted from all of the data in Figure 3A. It was, if anything, an erroneously high subtraction for the later incubation times when much of the methyl-^{14}C-AMe had been consumed.

In Figure 3B, 3.0 mM FH_4 is seen to function as a reducing agent for the rapid catalysis of N^5-methyl-^{14}C-FH_4-homocysteine transmethylation, pro-

vided AMe is added. The data (Fig. 3B) correspond to a turnover number of 300 mμmoles of methyl-^{14}C groups transferred to homocysteine/minute/mμmole of bound B_{12}. This turnover number is about half the turnover rate (500-780) which was observed for this same B_{12} enzyme preparation with $FMNH_2$ + DTT, instead of FH_4 + DTT, as the reducing system (3).

The data presented in Figures 1-3 support a carbonium ion mechanism (as proposed in Fig. 11 of Ref. 2) rather than a free radical mechanism for the catalysis of Reaction 1. We interpret the spectral changes in Figure 1 to be the first direct evidence for the participation of a B_{12s} enzyme in AMe dependent methionine synthesis and, moreover, the first time a B_{12s}-protein complex has been detected in corrinoid enzymology.

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References

1. Taylor, R.T., and Weissbach, H., J. Biol. Chem., 242 1502 (1967).
2. Taylor, R.T., and Weissbach, H., Arch. Biochem. Biophys., 129 745 (1969)
3. Taylor, R.T., and Hanna, M.L., manuscript submitted to Arch. Biochem. Biophys., December (1969).
4. Taylor, R.T., manuscript submitted to Arch. Biochem. Biophys., November (1969).
5. Taylor, R.T., and Weissbach, H., Arch. Biochem. Biophys., 129 728 (1969)
6. Bray, G.A., Anal. Biochem., 1 279 (1960).
7. Ertel, R., Brot, N., Taylor, R.T., and Weissbach, H., Arch. Biochem. Biophys., 126 353 (1968).
8. Boos, R.N., Carr, J.E., and Conn, J.B., Science, 117 603 (1953).
9. Hill, J.A., Pratt, J.M., and Williams, R.J.P., J. Theoret. Biol., 3 423 (1962).
10. Bonnet, R., Chem. Reviews, 63 573 (1963).
11. Rosenthal, S., Smith, L.C., and Buchanan, J.M., J. Biol. Chem., 240 836 (1965).