# FORMATION OF THE $N^5$-METHYLTETRAHYDROFOLATE-HOMOCYSTEINE METHYLTRANSFERASE HOLOENZYME FROM APOENZYME AND ADENOSYL-$B_{12}$*

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Summary: Two components of E. coli extract separable by DEAE-cellulose chromatography have been found to function together to convert the apoenzyme form of the $N^5$-methyltetrahydrofolate-homocysteine transmethylase and adenosyl-$B_{12}$ to holoenzyme in the presence of DPNH and homocysteine. They also form holoenzyme from hydroxo-$B_{12}$ and cyano-$B_{12}$ and constitute a DPNH-dependent reducing system that promotes the formation of methionine by holoenzyme.

When E. coli is grown in the absence of cobamides the apoenzyme form of $N^5$-methyltetrahydrofolate-homocysteine transmethylase accumulates (1). The formation of holoenzyme with this protein and various cobamides in vitro was investigated by Takeyama, Hatch and Buchanan (2) who reported that the conditions for the utilization of cyano-$B_{12}$ and adenosyl-$B_{12}$ were different.[1] Although a crude extract of E. coli that contained the apoenzyme was found to use the two cobamides for holoenzyme formation, a 35-45% ammonium sulfate fraction that utilized cyano-$B_{12}$ had little activity on the coenzyme. In subsequent studies, Brot and Weissbach (3) demonstrated that adenosyl-$B_{12}$ was converted to holoenzyme when protein of the 60-90% ammonium sulfate fraction of E. coli extract and DPNH were included in the incubation mixtures. They observed that this protein fraction and DPNH also permitted the utilization of hydroxy-$B_{12}$, but not cyano-$B_{12}$, and provided the reducing system required for the formation of methionine. All of these properties were attributed to an enzyme that they called "holoenzyme synthetase".

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[1]Abbreviations: cyano-$B_{12}$, cyanocobalamin; hydroxy-$B_{12}$, hydroxocobalamin; methyl-$B_{12}$, methylcobalamin; propyl-$B_{12}$, propylcobalamin; adenosyl-$B_{12}$, 5'-deoxyadenosylcobalamin; $CH_3$-$H_4$PteGlu, $N^5$-methyltetrahydropteroylglutamic acid; AMe, S-adenosyl-L-methionine iodide; DTE, dithioerythritol.

The present communication reports the isolation of two components of E. coli extract which together form holoenzyme from apoenzyme and adenosyl-$B_{12}$ in the presence of DPNH and homocysteine and describes some of their properties. The results of our studies on the mechanism of action of these substances will be presented in a later publication.

## Materials and Methods

Apoenzyme, obtained from E. coli W grown to late log phase by the procedure of Vogel and Bonner (4) was partially purified to remove DPNH-dependent reductase activity. All of the solutions used in the preparation of this protein fraction contained 0.002 M DL-homocysteine in addition to the constituents specified (2).

The E. coli W cells were broken by sonicating the organisms in 0.2 M K-$PO_4$ buffer, pH 7.45 (4 ml per g, wet weight) at 20 KC for 4 min at 0°. After centrifugation of the preparation and treatment of the supernatant solution with bovine pancreatic deoxyribonuclease I and ribonuclease (2), apoenzyme was precipitated by the addition of a dry K-$PO_4$ buffer salt mixture pH 7.45* (55 g/liter) followed by a similar mixture of $(NH_4)H_2PO_4$ and $(NH_4)_2HPO_4$** (191 g/liter). The precipitate was removed by centrifugation and dissolved in a minimum amount of 0.2 M K-$PO_4$ buffer, pH 7.45. Ten ml of this solution were chromatographed anaerobically (5) on a column of Sephadex G-200 (5 X 90 cm) equilibrated with the buffer. Fractions containing apoenzyme were identified by the presence of a precipitate of DL-homocystine after standing in air for 3 days at 0°. When protected with 0.002 M DL-homocysteine, the apoenzyme has been kept at 0-4° for several days and maintained in the frozen state at -15° for several months with relatively little loss in activity. The yield of apoenzyme was 70% of the material present in the dialyzed sonicated extract, and the increase in specific activity was 12-fold. Center fractions of the apoenzyme peak, which contained little DPNH-dependent reductase activity, were used in the experiments described in this communication.

The holoenzyme-forming enzyme system was isolated from the centrifugate

*185 g $K_2HPO_4 \cdot 3H_2O$ and 25.8 g $KH_2PO_4$.
**132 g $(NH_4)_2HPO_4$ and 13.8 g $(NH_4)H_2PO_4$.

obtained in the preparation of apoenzyme. Ammonium sulfate, 81 g/liter, was added to the solution, and the precipitate was removed by centrifugation. The active protein fraction was then salted out by further addition of ammonium sulfate, 181 g/liter. The precipitate was removed by centrifugation, dissolved in a minimum amount of 0.05 M K-$PO_4$ buffer pH 7.45, and dialyzed against the same buffer.

The two components of "holoenzyme synthetase" were separated by chromatography on DEAE cellulose.* One of them, Cp 1, was prepared by this procedure: the other component, Cp 2, was prepared more conveniently** from a heated extract (see below). One and seven tenths gram protein in 68 ml volume were introduced into a column of DEAE-cellulose* (4 X 40 cm) equilibrated with 0.05 M buffer, pH 7.45, and eluted with a linear gradient of K-$PO_4$ buffer, 0.05 M to 0.30 M, 1 liter total volume. Cp 1 activity associated with yellow material was eluted between 430 and 500 ml. The active portion of the eluate was concentrated with Sephadex G-25, and the protein was fractionated on a column of Sephadex G-100 (5 X 95 cm) with 0.1 M K-$PO_4$ buffer, pH 7.45. When 175 mg protein in 9.2 ml were chromatographed, Cp 1 was eluted between 1070 and 1170 ml. Active fractions were combined and concentrated with Sephadex G-25.

Cp 2, which is heat-stable, was prepared by bringing a suspension of the cells in 0.2 M K-$PO_4$ buffer (2 ml/g cells, wet weight) rapidly to 88° in a Pyrex double boiler with vigorous agitation. Heating at 88°-94° was continued for 10 min, then terminated by cooling the suspension immediately in ice-water. After centrifugation, the extract was treated with deoxyribonuclease I and ribonuclease (2), concentrated with a Diaflow PM-10 filter and dialyzed against 0.05 M K-$PO_4$ buffer, pH 7.45. One hundred and twenty-eight mg protein (corresponding to 19 g E. coli, wet weight) were chromatographed on a column of DEAE cellulose* (1.7 X 40 cm) equilibrated with 0.05 M K-$PO_4$ buffer, pH 7.45, with a linear gradient of the buffer 0.05 M-0.66 M, 600 ml total volume. Cp 2, eluted between 530 and 610 ml,

*Whatman DE-32.
**Much more Cp 2 can be placed on the column as heated extract.

was concentrated with Sephadex G-25, and fractionated on a column of Sephadex G-200 (1.7 X 42 cm) equilibrated with 0.2 M K-$PO_4$ buffer pH 7.43. Activity was eluted between 70 and 105 ml.

Gel filtration experiments indicated that Cp 1 and Cp 2 have molecular weights on the order of 70,000.

## Results and Discussion

As shown in Table 1, both Cp 1 and Cp 2 are required for holoenzyme formation with adenosyl-$B_{12}$. In this experiment apoenzyme was incubated with adenosyl-$B_{12}$, DPNH, DL-homocysteine, and Cp 1 and/or Cp 2. The amount of holoenzyme that was formed was determined by measuring the ability of the incubation mixture to form methionine when supplemented with propyl-$B_{12}$ to inhibit further holoenzyme formation (3) together with all of the cofactors and substrates that are required for the action of the holoenzyme. When both Cp 1 and Cp 2 were present during the

Table 1. Requirement for Cp 1 and Cp 2 in the formation of holoenzyme from apoenzyme and adenosyl-$B_{12}$.

| | Component of "holoenzyme synthetase" present in the incubation | | Methionine formed* |
|---|---|---|---|
| | First incubation | Second incubation | (nmoles) |
| 1. | Cp 1 | Cp 1 + Cp 2 | 2 |
| 2. | Cp 2 | Cp 1 + Cp 2 | 7 |
| 3. | Cp 1 + Cp 2 | Cp 1 + Cp 2 | 62** |

*Corrected for methionine formation in the absence of both Cp 1 and Cp 2 from the first incubation.

**The formation of holoenzyme in this preincubation was essentially complete. In a control lacking propyl-$B_{12}$, 72 nmoles methionine were formed.

The first incubation mixture contained: apoenzyme, 0.06 mg protein; DL-homocysteine, 10 μmoles; DPNH, 5 μmoles; adenosyl-$B_{12}$, 1 nmole; K-$PO_4$ buffer, pH 7.45, 100 μmoles; and, where indicated, Cp 1, 0.04 mg protein; and Cp 2, 0.14 mg protein. The total volume was 0.55 ml. Incubation was under $N_2$ for 30 min at 37°. In the second incubation the mixture contained the substances listed above, and propyl-$B_{12}$, 30 nmoles; $CH_3$-$H_4$PteGlu, 6.2 μmoles; AMe, 50 nmoles; mercaptoethanol, 10 μmoles; additional K-$PO_4$ buffer, 50 μmoles; and both Cp 1 and Cp 2. The total volume was 1 ml. Incubation was under $N_2$ for 2 h at 37°. Enzyme activity was terminated by the addition of 1 ml of 10% TCA, and methionine was determined microbiologically (6).

first incubation, holoenzyme was formed, as indicated by the production of methionine in the second. Little holoenzyme was formed when either Cp 1 or Cp 2 were omitted.

The results of the study shown in Table 2 are consistent with the hypothesis (3) that "holoenzyme synthetase" per se and DPNH provide a natural reducing system of the type that is required for methionine formation by holoenzyme. In the incubations described in the table apoenzyme and methyl-$B_{12}$ combined spontaneously (7, 1, 8) to form holoenzyme. The formation of methionine by the holoenzyme was greatly increased by the combination of Cp 1 and Cp 2. "Holoenzyme synthetase" is not the only DPNH-dependent reductase of E. coli that permits holoenzyme to form methionine. The ammonium phosphate fraction that contains apoenzyme formed methionine when incubated with methyl-$B_{12}$ and the substrates and cofactors listed in Table 2, although it contained no "holoenzyme synthetase". Chromatography of the fraction on Sephadex G-200 demonstrated that it contained at least two reductases of this type.

According to Brot and Weissbach (3), "holoenzyme synthetase" and DPNH form holoenzyme from apoenzyme and hydroxy-$B_{12}$ but not cyano-$B_{12}$. The results shown in Table 2 demonstrate that both of these cobamides may be converted to

Table 2. Requirement for Cp 1 and Cp 2 in the formation of methionine by apoenzyme and methyl-$B_{12}$, hydroxy-$B_{12}$, and cyano-$B_{12}$.

| Component of "holoenzyme synthetase" present | | Methionine formed (nmoles) | | |
|---|---|---|---|---|
| | | Methyl-$B_{12}$ | Hydroxy-$B_{12}$ | Cyano-$B_{12}$ |
| 1. | Cp 1 | 38 | 23 | 15 |
| 2. | Cp 2 | 8 | 10 | 0 |
| 3. | Cp 1 + Cp 2 | 150 | 88 | 95 |

The incubation mixture contained: apoenzyme (see below); Cp 1, 0.03 mg protein; and/or Cp 2, 0.02 mg protein; methyl-$B_{12}$, hydroxy-$B_{12}$, or cyano-$B_{12}$ 1 nmole; DL-homocysteine, 10 μmoles; DPNH, 5 μmoles; $CH_3$-$H_4$PteGlu, 6.2 μmoles; AMe, 50 nmoles; mercaptoethanol, 10 μmoles; and K-$PO_4$ buffer, pH 7.45, 170 μmoles. Two different apoenzyme preparations were employed, one (0.04 mg protein) in the study of methyl-$B_{12}$ and hydroxy-$B_{12}$, the other (0.1 mg protein) when cyano-$B_{12}$ was present. The total volume was 1 ml. Incubation was under $N_2$ for 2.5 h at 37°.

holoenzyme by the enzyme system that utilizes adenosyl-$B_{12}$.

Several proteins that have been briefly reported to play a role in methionine synthesis in E. coli may be identical with Cp 1 or Cp 2. Galivan and Huennekens (9) have indicated that they have separated the "methionine synthetase" of E. coli K12 into two proteins designated M and S. The two components formed methionine when incubated with methyl-$B_{12}$ (or hydroxy-$B_{12}$), $CH_3$-$H_4$PteGlu, homocysteine, AMe and DTE under argon. They also have isolated a DPNH-dependent reductase capable of participating in methionine synthesis that they consider to be the "holoenzyme synthetase" of Brot and Weissbach (3). It is not clear whether S is required when DTE is replaced by the reductase or whether the reductase can form holoenzyme from apoenzyme and adenosyl-$B_{12}$ in the presence of DPNH. Of interest also is the claim of Rüdiger and Jaenicke (10) that extracts of E. coli B contain a protein capable of demethylating the methyl-$B_{12}$ form of methionine synthetase (DM-factor). The substance S of Galivan and Huennekens (9) may be a monomeric form of Cp 2 and the DM-factor. All three substances are heat stable. Information concerning the reductase is insufficient to suggest whether it is Cp 1 or one of the DPNH-dependent reductases of E. coli that promote methionine synthesis in the absence of Cp 2.

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