BINDING OF SUBSTRATE TO N^5-METHYL-TETRAHYDROPTEROYLTRIGLUTAMATE-HOMOCYSTEINE TRANSMETHYLASE

Carolyn D. Whitfield* and Herbert Weissbach

Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014

Received November 22, 1968

Two enzymes in Escherichia coli catalyze the biosynthesis of methionine from homocysteine (4). One of these enzymes (B_{12}-transmethylase) is a cobamide containing protein which catalyzes methyl transfer from either N^5-methyltetrahydropteroylmonoglutamate (N^5-methyl-H_4-folate-G_1) or N^5-methyltetrahydropteroyltriglutamate (N^5-methyl-H_4-folate-G_3) to homocysteine in the presence of catalytic levels of S-adenosylmethionine and a reducing system (for review see reference 13). Recent studies from this laboratory (10) on the mechanism of methyl transfer catalyzed by the B_{12}-dependent enzyme have demonstrated that a methyl-B_{12} enzyme is an intermediate in this reaction.

The second enzyme (non-B_{12}-transmethylase) catalyzes methyl transfer from only N^5-methyl-H_4-folate-G_3 to homocysteine in the presence of magnesium and phosphate ions as described in the following reaction (3,5).

---

* This investigation was supported by a Public Health Service Predoctoral Fellowship (5 FO1 GM-29,891-04) from the National Institute of General Medical Sciences.

$$N^5\text{-methyl-}H_4\text{-folate-}G_3 + \text{homocysteine} \xrightarrow[P_i]{Mg^{++}} H_4\text{-folate-}G_3 + \text{methionine}$$

The present communication describes studies on the mechanism of methyl transfer catalyzed by the non-B_{12}-transmethylase, utilizing a nearly homogeneous preparation of the enzyme. Evidence is presented below for the formation of a stereospecific complex between the enzyme and its substrate, N^5-methyl-H_4-folate-G_3.

Previous studies of this reaction have been limited by the lack of substrate. In the present experiments, we are deeply indebted to Dr. Leon Ellenbogen of Lederle Laboratories for the generous gift of DL-pteroyltriglutamate (folate-G_3), which was originally synthesized by Boothe et al. (1). DL-Tetrahydropteroyltriglutamate (H_4-folate-G_3) was prepared by reduction of folate-G_3 with sodium borohydride (7), and DL-N^5-methyl-^{14}C-H_4-folate-G_3 was prepared essentially as described by Keresztesy and Donaldson (8). The N^5-methyl-^{14}C-H_4-folate-G_1 (18,000 cpm/mμmole) was obtained from Nuclear Chicago. The N^5-methyl-H_4-folate-G_3-homocysteine transmethylase was purified from E. coli K_{12} strain AB 1909, which was kindly supplied by Dr. Edward Adelberg. This organism is a methionine mutant, defective in N^5-N^{10}-methylene-H_4-folate reductase, and also requires arginine for growth. Growth to late log phase on minimal salt medium 56 (9), containing 0.5% glucose, 1×10^{-4} M D-methionine (Calbiochem) and 3×10^{-4} M L-arginine (Calbiochem) resulted in a 2 to 3-fold derepression of the enzyme in these cells compared

to wild type *E. coli* K_{12} or *E. coli* B cells. The enzyme which represented about 5% of the protein in the derepressed cells has been purified 15 to 20-fold and crystallized. The details of the purification procedure will be published in a separate communication. This enzyme preparation appeared to be nearly homogeneous by disc gel electrophoresis and ultracentrifugation. The high degree of purity of the enzyme was also supported by the results of a dual purification of the enzyme from cells repressed with L-methionine and cells derepressed with D-methionine. One unit of activity is that amount of enzyme which catalyzes the formation of 1 mμmole of methionine in 15 minutes at 37°C.

RESULTS AND DISCUSSION

As shown in Table I, the formation of methyl-^{14}C-methionine from N^5-methyl-^{14}C-H_4-folate-G_3 was dependent on the presence of homocysteine, enzyme, inorganic phosphate and was stimulated 2-fold by magnesium ion. N^5-Methyl-^{14}C-H_4-folate-G_1 could not replace the triglutamate folate derivative as methyl donor and dithiothreitol (DTT), mercaptoethanol and cysteine could not replace homocysteine. These dependencies are in agreement with those previously reported (3,5).

Evidence for an enzyme-substrate complex has been obtained using Sephadex G-50 chromatography (Fig. 1). Addition of the highly purified enzyme to DL-N^5-methyl-^{14}C-H_4-folate-G_3, followed by chromatography on Sephadex G-50, yielded a peak of radioactivity associated with the enzyme peak (elution volume

Table I

Requirements of the non-B_{12} transmethylase for methionine synthesis

Reaction Mixture	Methionine formed	
	cpm	μμmoles
Complete	2715	202
-Homocysteine	0	0
-Enzyme	0	0
-Na_2HPO_4	328	24.3
-Na_2HPO_4 + K_2HPO_4	2685	199
-Na_2HPO_4 + NaCl	280	20.8
-Na_2HPO_4 + Tris-Cl	220	16.3
-Mg^{++}	1331	98.6
-N^5-methyl-^{14}C-H_4-folate-G_3 + N^5-methyl-^{14}C-H_4-folate-G_1	0	0
-Homocysteine + either DTT or β-mercaptoethanol or cysteine	0	0

The complete system contained 3 mμmoles of N^5-methyl-^{14}C-H_4-folate-G_3 (13,500 cpm/mμmole); L-homocysteine, 50 mμmoles; Na_2HPO_4, pH 8.2, 500 mμmoles; magnesium acetate, 5 mμmoles; and purified enzyme (0.12 μg) in a total volume of 50 μl. After incubation for 15 minutes at 37°C, the methionine formed was assayed using a Dowex-1-Cl column procedure previously described (12).

The following were added where indicated: 3 mμmoles of N^5-methyl-^{14}C-H_4-folate-G_1 and 500 mμmoles of K_2HPO_4 pH 8.2, Tris-Cl pH 8.2, DTT, β-mercaptoethanol and L-cysteine. The pH (8.1) of the reaction mixture did not change when Na_2HPO_4 was omitted because of the buffering capacity of the other reaction components.

---

of 12-13 ml). No radioactivity was present in these fractions in the absence of enzyme. Complex formation did not occur when N^5-methyl-^{14}C-H_4-folate-G_1 was used in place of the tri-glutamate derivative.

Eighty per cent of the radioactivity associated with the enzyme-^{14}C complex (Fig. 1) was readily transferred to homocysteine to form methionine (Table II, part A). However,

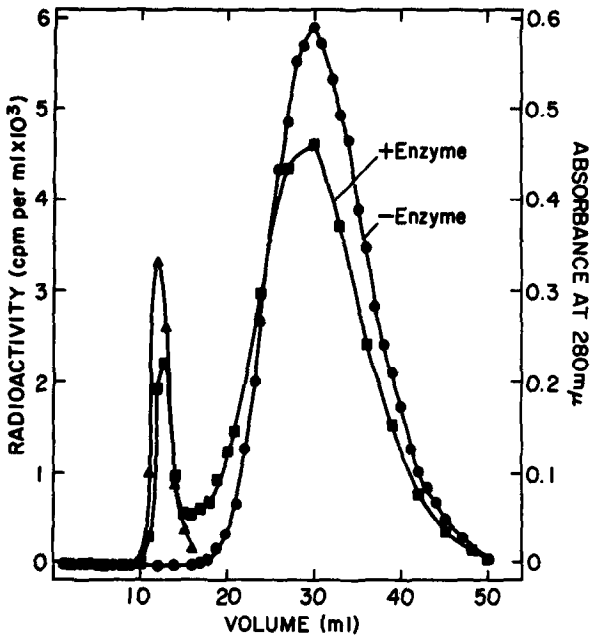

Figure 1. Isolation of an enzyme-^{14}C complex by Sephadex G-50 chromatography. ●, Radioactivity from N^5-methyl-^{14}C-H_4-folate-G_3 minus enzyme; ■, radioactivity from N^5-methyl-^{14}C-H_4-folate-G_3 plus enzyme; ▲, absorbance at 280 mμ of the enzyme. The reaction mixture contained N^5-methyl-^{14}C-H_4-folate-G_3, 5 mμmoles (75,000 cpm); potassium phosphate buffer, pH 7.8, 5 μmoles; magnesium sulfate, 250 mμmoles; DTT, 0.5 μmoles; and 0.5 mg of enzyme (1200 units) in a total volume of 70 μl. After 5 minutes at 0°C, the samples were diluted with 0.1 ml of H_2O and placed on a 1 cm x 30 cm (30 ml) Sephadex G-50 column, equilibrated with 0.01 M potassium phosphate buffer, pH 7.4. The column was eluted with this buffer (0.7 ml/min); 1 ml fractions were collected and assayed for radioactivity in a Packard Scintillation Spectrometer using a naphthalene dioxane counting fluid described by Bray (2).

since the triglutamate substrate was labeled in the methyl group, the radioactivity present with the enzyme (Fig. 1) could represent either a methylated enzyme or an enzyme-substrate complex containing an intact or modified substrate. In order to distinguish between these possibilities, the

radioactive fractions containing enzyme from Fig. 1 were heated at 65° for 15 minutes in the presence of 1 mM DTT. Although this treatment destroyed the endogenous enzymatic activity, control studies showed that it did not inactivate N^5-methyl-

Table II

Conversion of Enzyme-^{14}C Complex to Methionine

	Fraction Added	Methionine	% Conversion
		cpm	
A.	Unheated enzyme-^{14}C complex (840 cpm)		
	Complete	667	80
	-Homocysteine	45	5.4
B.	Heated enzyme^{14}C complex (1080 cpm)		
	Complete	811	75
	-Homocysteine	18	1.7
	-Enzyme	50	4.6
C.	Heated or Unheated DL-N^5-methyl-^{14}C-H_4-folate-G_3 (3000 cpm)		
	Complete	1080	36
	-Homocysteine	0	0
	-Enzyme	0	0

The incubation mixture contained L-homocysteine, 50 mμmoles; potassium phosphate buffer pH 7.8, 50 μmoles; magnesium sulfate, 2.5 μmoles; and DTT, 2.5 μmoles in a total volume of 0.5 ml.

In experiment A, the unheated enzyme-^{14}C complex (Fig. 1) was added to the incubation mixture. In B, the enzyme-^{14}C complex was heated for 15 minutes at 65°C in 1 mM DTT to destroy the enzymatic activity and the protein precipitate was removed by centrifugation. The supernatant and purified non-B_{12}-transmethylase, 24 units, were added to the incubation mixture. In C, heated or unheated DL-N^5-methyl-^{14}C-H_4-folate-G_3 and 24 units of non-B_{12} transmethylase were added to the incubation mixture. The reaction mixtures were incubated for 15 minutes at 37°C and then assayed for methionine (12). The ^{14}C product was also identified as methionine as previously described (11). In all experiments, the reactions went to completion.

H_4-folate-G_3. The heated fractions were either examined using paper chromatography (see below), or were incubated with active non-B_{12}-transmethylase in the presence of homocysteine, phosphate and magnesium ions. In the latter case, any enzymatic formation of methionine would indicate that the intact substrate had been present in the complex while a modified substrate, or methylated enzyme would not be expected to yield methionine. As seen in Table II, part B, 75% of the radioactivity in the heated complex was recovered in methionine after the enzymatic incubation. Experiment C in Table II, is a control using either heated or unheated DL-N^5-methyl-^{14}C-H_4-folate-G_3 in place of the heated complex. It can be seen that only 36% of the initial folate substrate was enzymatically converted to methionine. These data suggest that the enzyme-^{14}C complex contains the active stereoisomer of the folate substrate.

Ascending paper chromatography in two solvent systems (1 M Na_2HPO_4, β-mercaptoethanol, water [42:1:57] [6]; ethanol, ammonium hydroxide, β-mercaptoethanol, water [45:5:1:49]) of the heated enzyme-^{14}C complex revealed a single radioactive peak corresponding to the R_f value of similarly treated N^5-methyl-^{14}C-H_4-folate-G_3.

In contrast to the results obtained with the B_{12}-transmethylase (10), no evidence for the formation of a methylated enzyme has been obtained with the non-B_{12}-transmethylase. Instead, a stereospecific binding of the intact folate substrate to the enzyme has been demonstrated.

REFERENCES

1. Boothe, J.H., Semb, J., Waller, C.W., Angier, R.B., Mowat, J.H., Hutchings, B.L., Stokstad, E.L.R., and SubbaRow, Y., J. Amer. Chem. Soc. 71, 2304 (1949).
2. Bray, G.A., Anal. Biochem. 1, 279 (1960).
3. Buchanan, J.M., Elford, H.L., Loughlin, R.E., McDougall, B.M., and Rosenthal, S., Ann. N.Y. Acad. Sci. 112, 756 (1964).
4. Foster, M.A., Tejerina, G., Guest, J.R., and Woods, D.D., Biochem. J. 92, 476 (1964).
5. Guest, J.R., Friedman, S., Foster, M.A., Tejerina, G., and Woods, D.D., Biochem. J. 92, 497 (1964).
6. Gupta, V.S., and Huennekens, F.M., Arch. Biochem. Biophys. 120, 712 (1967).
7. Hillcoat, B.L., and Blakeley, R.L., Biochem. Biophys. Res. Commun. 15, 303 (1964).
8. Keresztesy, J.C., and Donaldson, K.O., Biochem. Biophys. Res. Commun., 5, 286 (1961).
9. Monod, J., Cohen-Bazire, G., and Cohn, M., Biochim. Biophys. Acta 7, 585 (1951).
10. Taylor, R.T., and Weissbach, H., Arch. Biochem. Biophys. 123, 109 (1968).
11. Taylor, R.T., Whitfield, C., and Weissbach, H., Arch. Biochem. Biophys. 125, 240 (1968).
12. Weissbach, H., Peterkofsky, A., Redfield, B.G., and Dickerman, H., J. Biol. Chem. 238, 3318 (1963).
13. Weissbach, H., and Taylor, R.T., Fed. Proc. 25, 1649 (1966).