Short Communications

The Substrate Specificity of 5-Methyltetrahydropteroyltriglutamate-Homocysteine Methyltransferase

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The presence in *Escherichia coli* extracts of a B₁₂-independent methyltransferase capable of methylating homocysteine with methyl-H₄-PteGlu₃* was demonstrated by D. D. Woods and his associates (Guest, Friedman, Foster, Tejerina & Woods, 1964b). Similar enzymes have been reported to occur in other micro-organisms (Woods, Foster & Guest, 1964; Morningstar & Kisliuk, 1965; Cauthen, Foster & Woods, 1966; Burton & Sakami, 1969) and in higher plants (Woods *et al.* 1964; Burton & Sakami, 1969). The enzyme of *E. coli* was reported to be specific for folates containing at least three glutamic acid residues (Guest, Friedman, Dilworth & Woods, 1964a) and therefore was termed a 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase.

In the interpretation of the results of our experiments on methionine-requiring strains of *Neurospora crassa* blocked between homocysteine and methionine, the ability of the organism to methylate homocysteine with methyl-H₄-PteGlu₂ was of importance. *N. crassa* possesses only the B₁₂-independent methyltransferase. Although Woods and his associates (Guest *et al.* 1964a) had claimed that this enzyme does not act on methyl-H₄-PteGlu₂ they had not presented the results of their studies of this folate. For this reason methyl-H₄-PteGlu, methyl-H₄-PteGlu₂ and methyl-H₄-PteGlu₃ were synthesized chemically, and the transmethylation of these substances with homocysteine in extracts of *N. crassa* was investigated. The specificities of the methyltransferases of *E. coli* and *Saccharomyces cerevisiae* were also investigated.

Materials and methods. The folates methyl-H₄-PteGlu, methyl-H₄-PteGlu₂ and methyl-H₄-PteGlu₃ were prepared from PteGlu, PteGlu₂ and PteGlu₃ respectively. PteGlu was a commercial product; PteGlu₂ and PteGlu₃ were synthesized by the method of Boothe *et al.* (1949). The mono-, di- and

tri-glutamate forms of methyltetrahydrofolate were prepared by hydrogenation of the folates (Jones, Guest & Woods, 1961) followed by methylation of the tetrahydrofolates and purification of the methyltetrahydrofolates as described for the monoglutamate by Sakami (1963). The identity of the methyl-H₄-PteGlu₂ and the absence of contamination with the triglutamate was demonstrated by comparison of the elution pattern of the compound on an analytical DEAE-Celite column (Silverman, Law & Kaufman, 1961) with that of methyl-H₄-PteGlu₃ and of methyl-H₄-PteGlu₂ prepared by the action of chicken pancreas conjugase on the triglutamate (Kazenko & Laskowski, 1948).

N. crassa wild-type, RL21a, was grown at 30° for 36 hr. with aeration on Vogel's (1956) minimal medium supplemented with 2% (w/v) of sucrose. After harvesting, the mycelia were washed with water, suspended in 1 part (w/v) of 0.1 M-potassium phosphate buffer, pH 7.0, and passed through a Hughes press. The suspension was centrifuged for 30 min. at 27 000g and the supernatant solution was passed through a Sephadex G-25 column equilibrated with 0.1 M-potassium phosphate buffer, pH 7.0. The protein fraction obtained from the column was used for enzyme assay without further treatment. The serine-glycine-requiring mutant of *E. coli*, PA-15, was grown on Vogel's (1956) medium supplemented with 0.08% of glycine and 0.4% of glucose. After harvesting, the paste of *E. coli* was mixed with 3 parts (w/v) of 0.1 M-potassium phosphate buffer, pH 7.8, and passed through a Hughes press. The suspension was treated with deoxyribonuclease I as described by Hatch, Larrabee, Cathou & Buchanan (1961) to decrease the viscosity and was then centrifuged for 30 min. at 27 000g. The supernatant solution was passed through a Sephadex G-25 column equilibrated with 0.1 M-potassium phosphate buffer, pH 7.8, and the protein fraction was used for enzyme assay without further treatment. A B₁₂-independent trans-methylase of *S. cerevisiae* was purified by the procedure of Burton & Sakami (1969).

Two types of incubation mixtures were used in

* Abbreviations for members of the folic acid group: PteGlu, pteroylmonoglutamate; PteGlu₂, pteroyl- γ -glutamylglutamate; PteGlu₃, pteroyl- γ -glutamyl- γ -glutamylglutamate; methyl-H₄, the 5-methyl-tetrahydro derivative of the above compounds; folate is used in general reference to all members of the group.

Table 1. Activity of *B*₁₂-independent methyltransferase on different forms of methyltetrahydrofolate

Details of the assay conditions for each type of transmethyrase are given under Materials and methods.

	L-Methionine formed (μmoles/hr./mg. of protein)			10 ³ <i>K</i> _m (M)	
	<i>N. crassa</i>	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>N. crassa</i>	<i>S. cerevisiae</i>
5-Methyltetrahydrofolate present					
Methyl-H ₄ -PteGlu	0.006	0.001	0.066	—	—
Methyl-H ₄ -PteGlu ₂	0.432	0.135	16.3	0.96	0.43
Methyl-H ₄ -PteGlu ₃	0.402	0.113	17.0	0.82	0.38

measuring the activity of the methyltransferases prepared as described above. With the extract of *N. crassa* and the purified enzyme of *S. cerevisiae* the incubation mixture contained: L-homocysteine, 1.5 μmoles; β-mercaptoethanol, 60 μmoles; potassium phosphate buffer, pH 7.0, 30 μmoles; *N. crassa* extract, 0.25 mg. of protein, or *S. cerevisiae* enzyme, 4.5 μg. of protein; 5-methyltetrahydrofolate, 1.0 μmole. The total volume was 0.3 ml. Incubation was aerobic at 37° for 30 min. Tetrahydrofolate was determined by the procedure of Rosenthal, Smith & Buchanan (1965). In this methyl transfer reaction L-methionine is formed in amounts equivalent to the tetrahydrofolate.

When the extract of *E. coli* was employed the incubation mixture contained: L-homocysteine, 5 μmoles; β-mercaptoethanol, 10 μmoles; potassium phosphate buffer, pH 7.8, 50 μmoles; *E. coli* extract, 1.1 mg. of protein; methyl-H₄-PteGlu, 1.0 μmole, or methyl-H₄-PteGlu₂, 1.0 μmole, or methyl-H₄-PteGlu₃, 0.75 μmole. The total volume was 0.5 ml. Incubation was aerobic at 37° for 60 min. The reaction was stopped with trichloroacetic acid and methionine was determined microbiologically with *Leuconostoc mesenteroides* P-60 (Steel, Sauberlich, Reynolds & Baumann, 1949).

Results. The methyltransferase activity of the preparations is shown in Table 1. With the crude extract of *N. crassa* both the di- and tri-glutamate forms of methyltetrahydrofolate were transmethyated, but not the monoglutamate. Similar results were obtained with a crude extract of *E. coli* PA-15 and a homogeneous enzyme obtained from *S. cerevisiae* (Burton & Sakami, 1969). The results obtained with the purified yeast protein which was homogeneous as determined by ultracentrifugation and disc electrophoresis, indicate that the di- and tri-glutamate forms of methyltetrahydrofolate are transmethyated by the same enzyme.

The transmethylation of methyl-H₄-PteGlu₂ did not involve conversion of this compound into methyl-H₄-PteGlu₃ and transmethylation of the triglutamate. The large amount of methionine formed in these incubations would have required

the formation of a substantial amount of tri-glutamate. Neither glutamate nor an energy source sufficient to account for this synthesis was added to, or could have been present in, our enzyme preparations. The yeast methyltransferase was a pure protein and the *E. coli* and *N. crassa* extracts had been freed of substrates and dissociable coenzymes by passage through a column of Sephadex G-25.

The specificity of the B₁₂-independent methyltransferase for a polyglutamate form of methyltetrahydrofolate is concerned at least in part with a requirement for two glutamic acid residues in the binding of substrate to the enzyme. The demonstration by Guest & Woods (1965) that methyl-H₄-PteGlu is not only not transmethyated itself, but also does not inhibit the transmethylation of the triglutamate, makes it clear that the monoglutamate does not bind to the enzyme. The addition of a third glutamic acid residue to the diglutamate does not greatly improve the binding. In our experiments the *K*_m values for the di- and tri-glutamate were similar for both the yeast and *Neurospora* enzymes, as shown in Table 1.

Since the B₁₂-independent methyltransferase has now been shown to transmethyate the di-, tri- and probably the hepta-glutamate (Jones *et al.* 1961) forms of methyltetrahydrofolate, it is suggested that the enzyme be called 5-methyltetrahydropteroylpolyglutamate-homocysteine methyltransferase, rather than 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase as proposed by Woods and his associates.

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