DEMONSTRATION OF SEPARATE BINDING SITES FOR THE FOLATE COENZYMES AND DEOXYNUCLEOTIDES WITH INACTIVATED LACTOBACILLUS CASEI THYMIDYLATE SYNTHETASE

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SUMMARY

In contrast to (+)5,10-methylenetetrahydropteroylmonoglutamate which does not bind to Lactobacillus casei thymidylate synthetase, the corresponding tetraglutamate analog binds to a single site with a $K_D = 2 \times 10^{-5}$ M. Alkylation of one of the enzyme's four cysteines with N-ethylmaleimide or iodoacetate prevented the binding of dUMP, but did not affect the binding of the pteroyltetraglutamate. Inactivation of the synthetase with carboxypeptidase A, however, prevented the binding of (+)5,10-methylenetetrahydropteroyltetraglutamate but not that of dUMP. The binding of (+)5,10-methylenetetrahydropteroyltetraglutamate to native enzyme was associated with the appearance of a positive circular dichroic band at 303 nm ($[\theta] = 7 \times 10^4$ deg·cm^{2}dmol^{-1}). The latter effect was not impaired by the inhibition of the enzyme with N-ethylmaleimide, whereas formation of the ternary complex, coenzyme-synthetase-FdUMP, was prevented by alkylation. These studies reveal that thymidylate synthetase can be inactivated in a manner that does not prevent the binding of the substrates individually.

INTRODUCTION

Previous studies on thymidylate synthetase (1,2) have shown that the Lactobacillus casei enzyme readily binds one mol of dUMP per mol of dimeric enzyme (M.W. = 70,000) (3), but that 5,10-CH_2H_4PteGlu[1] binding does not occur in the absence of added deoxynucleotides when examined by either equilibrium dialysis (1) or circular dichroism (4). Since the folates of L. casei are largely in the

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[1]Abbreviations are: PteGlu, Folic acid; (+)5,10-CH_2H_4PteGlu, (+)5,10-methylenetetrahydrofolate; (+)5,10-CH_2H_4PteGlu$_4$, (+)5,10-methylenetetrahydropteroyltetraglutamate; NEM, N-ethylmaleimide; IAA, iodoacetic acid; FdUMP, 5-fluoro-2'-deoxyuridylate; 4-N-OH-dCMP, 4-N-hydroxy-2'-deoxycytidylate; dUMP, 2'-deoxyuridylate.

form of the tetraglutamate with lesser amounts of the tri and penta (5), and the earlier studies employed the mono, a form undetected in L. casei, we undertook a study to determine the interaction of (+)5,10-$CH_2H_4PteGlu_4$ with the L. casei synthetase.

MATERIALS AND METHODS

Thymidylate synthetase was prepared as described previously as were FdUMP and [2-^{14}C]FdUMP (1,4). The preparation and purification of $PteGlu_4$ and its corresponding [^{14}C]-glutamic acid derivative have been described (6,7). This compound was converted to the corresponding dihydro-derivative by reduction with sodium dithionite (8). The biologically active isomer of $H_4PteGlu_4$ was synthesized enzymatically (9) with L. casei dihydrofolate reductase and purified by the method of Zakrzewski and Sansone (10). (-)$H_4PteGlu_4$ was converted to the (+)5,10-methylene derivative prior to use by the addition of formaldehyde. The concentration of the resulting product was determined as described previously (1) and the radioactive derivative was found to have a specific activity of 1.65×10^6 dpm/μmol. IAA was recrystallized from ethyl acetate-petroleum ether. The concentration of NEM (recrystallized from ethanol-water) was determined spectrophotometrically ($Em_{302} = 620\ M^{-1}cm^{-1}$) (11). Equilibrium dialysis (1) and circular dichroic studies (4) were conducted as described earlier. NEM-inhibited enzyme was prepared from thiol-free enzyme by reacting the enzyme with 1.2 mol of NEM/mol enzyme in 0.1 M Tris·HCl, pH 8.15, for 90 minutes at 25°C under an argon atmosphere. Enzyme containing a single mol of S-carboxymethylcysteine per mol of enzyme was prepared as described (12). Both NEM and IAA-reacted synthetase preparations were totally devoid of enzymatic activity following alkylation. Inactive enzyme was also obtained by treating 1 mg of thymidylate synthetase with 50 μg of bovine carboxypeptidase A[2] in 0.2 ml of 0.05 M potassium phosphate and 0.02 M mercaptoethanol at 25°C until all synthetase activity was lost (ca. 30 min) (13).

RESULTS AND DISCUSSION

Equilibrium dialysis was employed as the most sensitive method for determining the number of binding sites and dissociation constants that result from the interaction of thymidylate synthetase and (+)5,10-$CH_2H_4PteGlu_4$. By this procedure, it was determined that the enzyme bound 1 mol of (+)5,10-$CH_2H_4PteGlu_4$ per mol of enzyme (Fig. 1), a result which contrasts with the previously reported finding (1) that 5,10-CH_2H_4PtGlu is not bound unless FdUMP or 4-N-hydroxy dCMP is present, also (1). The higher affinity of the polyglutamate derivatives could be inferred from the studies of Kisliuk et al. (14) who found that these compounds have a lower K_m and higher V_{max} than (+)5,10-$CH_2H_4PteGlu$ when compared as substrates for thymidylate synthetase. The single site binding

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[2]Kindly provided by Dr. T. H. Plummer, Jr.

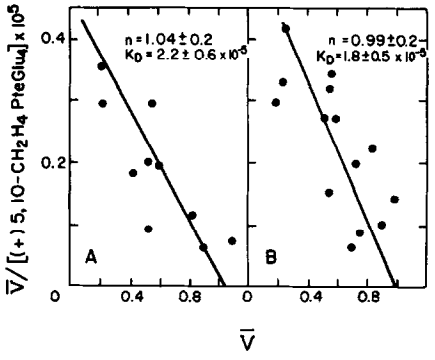

Figure 1. Binding of (+)5,10-$CH_2H_4PteGlu_4$ to L. casei thymidylate synthetase as measured by equilibrium dialysis. (A) 50 mM potassium phosphate (pH 7.0) and 20 mM 2-mercaptoethanol. (B) 50 mM Tris·HCl (pH 7.0), 20 mM $MgCl_2$ and 20 mM 2-mercaptoethanol. In both (A) and (B), the ligand side contained (+)5,10-CH_2H_4-$PteGlu_4$ at concentrations which varied from 0.01 to 0.25 mM and formaldehyde was present at 3.0 mM. The protein side contained 1 nmol (0.07 mg) of synthetase and the final volume on each side of the membrane was 60 μl. Following 4 hours of dialysis, the samples were removed and duplicate 20-μl samples were counted. All other experimental details have been described (1). Also see Materials and Methods.

shown for (+)5,10-$CH_2H_4PteGlu_4$ is in accord with other studies (1,2) indicating that the L. casei thymidylate synthetase although composed of two identical subunits (3,15) contains only one binding site for its substrates when each is measured in the absence of the other. In the presence of FdUMP however, two binding sites were obtained for 5,10-$CH_2H_4PteGlu_4$ (Table 1) as was shown previously for 5,10-$CH_2H_4PteGlu_1$ (1). As indicated, 4-N-HO-dCMP, a competitive inhibitor of the synthetase, yielded an anticipated value of one binding site for both folate derivatives (1). The binding of the folate analog differs from deoxynucleotide binding (1) in that the affinity of (+)5,10-$CH_2H_4PteGlu_4$ was the same in Tris·HCl and potassium phosphate (Fig. 1A and B, K_D = 2×10^{-5} M). As indicated earlier (1) the affinity of the enzyme for dUMP or FdUMP is greatly reduced in phosphate buffer.

It has been shown that thymidylate synthetase is inactivated by reaction of one of its four cysteines with a sulfhydryl reagent and that this effect can be prevented by the presence of dUMP (2,3,12,16,17). In view of the binding

Table 1. Effect of nucleotides on the binding of (+)5,10-CH_2H_4PteGlu and (+)5,10-CH_2H_4PteGlu$_4$ as shown by equilibrium dialysis

COMPOUND	DEOXYNUCLEOTIDE ADDED	BINDING SITES	$K_D \times 10^6$
(+)5,10-CH_2H_4PteGlu	none	none	-
	4-N-OH-dCMP	1.1	1.9
	FdUMP	1.82	<0.01
(+)5,10-CH_2H_4PteGlu$_4$	none	0.99	18
	4-N-OH-dCMP	1.0	<0.01
	FdUMP	1.6	<0.01

The binding measurements were conducted as described in Fig. 1 using 50 mM Tris·HCl and 20 mM $MgCl_2$ (pH 7.0). When present, 4-N-OH-dCMP and FdUMP were included on both sides of the membrane at 0.16 mM and 0.08 mM, respectively. The specific activity of (+)5,10-CH_2H_4[2-^{14}C]PteGlu was 3.0×10^6 dpm/μmol; that of (+)5,10-CH_2H_4Pte[^{14}C]-GluGlu$_3$ is in Materials and Methods.

of (+)5,10-CH_2H_4PteGlu$_4$ to the enzyme in contrast to its monoglutamate derivative, it was now possible to determine if alkylation affects the binding of each substrate differently. The enzyme was completely inactivated by reacting with one mol of NEM or one of IAA and the extent of binding was measured by equilibrium dialysis (Table 2). The specificity of the NEM alkylation reaction is indicated by the fact that the inhibition could be prevented by dUMP at ratios of NEM to enzyme of greater than 2 to 1. As expected, reaction of one of the enzyme's cysteines prevented the binding of dUMP and FdUMP. Surprisingly though, the binding of (+)5,10-CH_2H_4PteGlu$_4$ to enzyme that had been pretreated with IAA or NEM was not impaired in the former case and only slightly in the latter. Although not shown here, the NEM-treated enzyme could not form a stable ternary complex composed of 5,10-CH_2H_4PteGlu$_4$-enzyme-FdUMP, when determined with [2-^{14}C]FdUMP as a probe.

The demonstration that carboxypeptidase A inactivates thymidylate synthetase

Table 2. Effect of alkylation and carboxypeptidase A treatment on substrate binding of *Lactobacillus casei* thymidylate synthetase

COMPOUND TESTED	TREATMENT	BINDING SITES	$K_D \times 10^5$
(+)5,10-CH_2H_4PteGlu$_4$	IAA	1.0 ± 0.3	2.5 ± 1.0
dUMP	IAA	ndb[a]	
(+)5,10-CH_2H_4PteGlu$_4$	NEM	0.83 ± 0.2	4.0 ± 1.7
dUMP	NEM	ndb	
FdUMP	NEM	ndb	
(+)5,10-CH_2H_4PteGlu$_4$	Carboxypeptidase A	ndb	
dUMP	Carboxypeptidase A	1.1 ± 0.09	0.51 ± 0.05
(+)5,10-CH_2H_4PteGlu$_4$	Carboxypeptidase A + NEM	ndb	
dUMP	Carboxypeptidase A + NEM	ndb	

Equilibrium dialysis was conducted as in Fig. 1 using the 50 mM Tris·HCl, 20 $MgCl_2$ (pH 7.0) buffer system. Enzyme inactivation with NEM, IAA and carboxypeptidase A is described in Materials and Methods. When the binding of [2-^{14}C]dUMP (2.2×10^7 dpm/μmol) was measured, its concentration was varied between 0.002 and 0.07 mM; [2-^{14}C]FdUMP (6.2×10^6 dpm/μmol) was varied between 0.005 and 0.3 mM. For specific activity of (+)5,10-CH_2H_4Pte[^{14}C]GluGlu$_3$, see Materials and Methods.

[a]ndb = no detectable binding.

by removing a single terminal valine residue (13) provided a means of determining whether this treatment alters the capacity of the synthetase to bind either dUMP or (+)5,10-$CH_2H_4PteGlu_4$. In the case of dUMP, binding was essentially unaltered, but that of (+)5,10-$CH_2H_4PteGlu_4$ was completely blocked (Table 2). This effect is in contrast to that observed above with enzyme that had been inactivated with alkylating agents. Sequential treatment of the enzyme with carboxypeptidase A and then NEM resulted in the loss of both folate and deoxynucleotide binding sites (Table 2). Inactivation of the enzyme by heating at 55°C for 5 min also prevented the binding of either substrate.

The binding of (+)5,10-$CH_2H_4PteGlu_4$ to thymidylate synthetase was also examined by circular dichroism. We have shown previously that the interaction between various PteGlu analogs and the enzyme occurs only in the presence of deoxynucleotides and is reflected by a positive ellipticity band at about 305 nm (4). This effect results from an enhancement of the enzyme's affinity for pteroylmonoglutamate derivatives by specific deoxynucleotides (1). In the case of (+)5,10-$CH_2H_4PteGlu_4$, an increase in ellipticity in the region of 303 nm was observed when (+)5,10-$CH_2H_4PteGlu_4$ alone was added to the enzyme (Fig. 2A). If corrected for the contribution of the enzyme, the molar ellipticity at saturation is 7×10^4 deg·cm^{2}dmol^{-1} (303 nm). Addition of FdUMP to enzyme saturated with (+)5,10-$CH_2H_4PteGlu_4$ yielded a spectrum with a positive ellipticity band at 305 nm, a broad positive band between 340 and 450 nm, and negative ellipticity bands at 284 and 332 nm (Fig. 2B). This spectrum is nearly identical with that reported previously for the ternary complex of (+)5,10-CH_2H_4PteGlu-enzyme-FdUMP (4), and corroborated recently by Donato et al. (18). It should be noted that the intensity of the 305 nm CD band is slightly greater when the tetraglutamate derivative ($[\theta] = 11 \times 10^4$ deg.cm^{2}dmol^{-1}) replaces the monoglutamate ($[\theta] = 7 \times 10^4$ deg·cm^{2}dmol^{-1}) in the ternary complex.

The inability of NEM to prevent the binding of (+)5,10-$CH_2H_4PteGlu_4$ to the enzyme was also substantiated by the CD data (Fig. 2C). A comparison with Fig. 2A reveals that increasing amounts of (+)5,10-$CH_2H_4PteGlu_4$ effected an

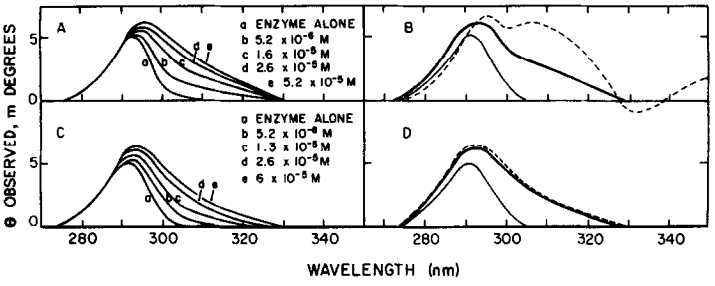

Figure 2. The binding of (+)5,10-CH_2H_4PteGlu$_4$ to L. casei thymidylate synthetase as demonstrated by circular dichroism. CD measurements were conducted as described previously (4). Each cuvette contained thymidylate synthetase, 12 nmol (0.84 mg); 2-mercaptoethanol, 20 mM; potassium phosphate (pH 7.0), 50 mM; and formaldehyde, 0.5 mM; and in (A) and (C), increasing amounts of (+)5,10-CH_2H_4PteGlu$_4$ (b through c). The total volume was 2 ml. The same solvent was used in (B) and (D) as in (A) and 100 nmol of (+)5,10-CH_2H_4PteGlu$_4$ (——) was added to the enzyme (——) followed by 60 nmol of FdUMP (----). NEM-inactivated enzyme was employed in (C) and (D). In all cases, the spectrum was corrected for the contribution of (+)5,10-CH_2H_4PteGlu$_4$, which possesses a CD spectrum nearly identical to that of (+)5,10-CH_2H_4PteGlu (4).

identical increase in the CD spectrum although the enzyme had been completely inactivated by NEM. However, this binary complex cannot react with FdUMP to form a spectrum characteristic of the ternary complex obtained with active enzyme (Fig. 2B). No further change in the CD spectrum of the binary complex was observed even with a 5-fold excess of FdUMP (Fig. 2D). It is evident that while binary complex formation with the appropriate ligand can occur with NEM inactivated synthetase, ternary complexes do not occur on addition of the second ligand.

Evidence is provided in these studies for a clear distinction between the binding sites for (+)5,10-CH_2H_4PteGlu$_4$ and either dUMP or FdUMP. Cysteine is strongly implicated in the deoxynucleotide binding site, since alkylation of this amino acid prevents binding of the deoxynucleotides without altering the binding of (+)5,10-CH_2H_4PteGlu$_4$. More direct evidence for the role of cysteine was obtained recently by sequencing the FdUMP-containing peptide from thymidylate synthetase which revealed that FdUMP is covalently bound to cysteine (12).

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