Factors Affecting Substrate Binding in *Lactobacillus casei* Thymidylate Synthetase as Studied by Equilibrium Dialysis†

John H. Galivan, Gladys F. Maley, and Frank Maley*

ABSTRACT: The binding of deoxynucleoside 5′-monophosphates and various folate derivatives to *Lactobacillus casei* thymidylate synthetase was measured by equilibrium dialysis. The substrate, deoxyuridylate (dUMP), and the product, thymidylate (dTMP), were bound to the enzyme at a ratio of unity and appeared to compete for the same site. The binding of each was tighter in 50 m*M* Tris-HCl (pH 7.1) than in 50 m*M* potassium phosphate (pH 7.0). Folate derivatives increased the affinity of the enzyme for the substrate to a greater extent than for the product, although they themselves did not appear to be bound in the absence of substrate or substrate analogues. However, in the presence of enzyme, dUMP or 4-*N*-OH-dCMP, and either 7,8-dihydrofolate or methotrexate, a ternary complex was obtained, with the folate derivatives exhibiting single site binding. The binding of dUMP in the ternary complex was 25-fold greater than that of 7,8-dihydrofolate and 50-fold greater than that of methotrexate. Supporting evidence for the enhanced stability of the ternary complex was provided by heat inactivation studies. As in the case of deoxynucleotide binding to the synthetase, the ternary complex was more stable in Tris-HCl than in potassium phosphate buffer. The binding characteristics of the substrate analogue 5-fluoro-2′-deoxyuridylate (FdUMP) could be clearly distinguished from that of dUMP by comparing their binding in phosphate and Tris-HCl. While each deoxynucleotide exhibited only single site binding in phosphate, a second site was clearly demonstrated for FdUMP with Tris-HCl. The binding of FdUMP to each site appeared to be equal in the presence of methotrexate or (−)5,10-methylene tetrahydrofolate and was increased about 17-fold in Tris-HCl. Two sites were also obtained for FdUMP in the presence of 7,8-dihydrofolate, but Scatchard analyses revealed a biphasic curve, with the second site possessing a higher dissociation constant than the first. A second low affinity FdUMP binding site was also detected in phosphate buffer when 7,8-dihydrofolate or (−)5,10-methylene tetrahydrofolate was included in the binding assay. In the presence of (+)5,10-methylene tetrahydrofolate, however, 2 mol of FdUMP was bound stoichiometrically to 1 mol of enzyme regardless of the buffer used. The significance of these results is discussed in relation to the presence of two apparently identical subunits in the native enzyme.

Studies on the mechanism of one carbon transfer by thymidylate synthetase and the inhibition of this enzyme by FdUMP[1] have been greatly facilitated by the increased availability of crystalline enzyme from *Lactobacillus casei* (Dunlap et al., 1971; Leary and Kisliuk, 1971; Galivan et al., 1975). Santi and McHenry (1972) and Langenbach et al. (1972) observed that a stable ternary complex containing 2 mol of FdUMP/mol of enzyme (Santi et al., 1974) is formed from enzyme, 5,10-CH_2H_4folate, and FdUMP. This ratio corresponds to the amount of FdUMP required to inhibit the enzyme stoichiometrically in the presence of 5,10-CH_2H_4folate (Santi and McHenry, 1972; Santi et al., 1974; Galivan et al., 1974). Other studies employing circular dichroism (Galivan et al., 1975), disc gel electrophoresis (Aull et al., 1974b), and ultraviolet spectroscopy (Aull et al., 1974c) have shown that 5,10-CH_2H_4folate also binds to the enzyme at a maximal ratio of 2:1 in the presence of FdUMP. This ternary complex is apparently stable to sodium dodecyl sulfate disc gel electrophoresis (Langenbach et al., 1972), to denaturation (Santi et al., 1974; Danenberg et al., 1974; Galivan et al., 1974), and to proteolysis (Santi et al., 1974, Danenberg et al., 1974) suggesting that the ligands are covalently linked to the enzyme (Santi et al., 1974; Danenberg et al., 1974). Since the native enzyme is a dimer composed of two apparently identical subunits (mol wt 35 000) (Dunlap et al., 1971; Loeble and Dunlap, 1972), it is tempting to postulate the presence of one FdUMP binding site/subunit. It should be noted, however, that the synthetase is completely inactivated by reaction of only one of the enzyme's four sulfhydryl groups with *p*-hydroxymercuribenzoate (Dunlap et al., 1971) suggesting that there are more FdUMP binding sites than catalytic sites or that the sulfhydryl reagent and FdUMP sites are not identical. The latter proposal was presented recently by McHenry and Santi (1974).

Ternary complex formation between FdUMP, thymidylate synthetase, and 5,10-CH_2H_4folate (Langenbach et al., 1972; Santi and McHenry, 1972) is tighter than that formed in the presence of other folate analogues (Danenberg et al., 1974; Galivan et al., 1974; 1975; Santi et al., 1974). The interaction of dUMP alone with thymidylate synthetase has been assumed from its capacity to protect the enzyme against heat inactivation (Lorenson et al., 1967; Bonney and Maley, 1975). This finding was also verified for dUMP with the *L. casei* enzyme (Leary and Gaumont, 1973) and for FdUMP (Galivan et al., 1975) by means of circular dichroism. In a more detailed study, Leary et al. (1975) found that 1 mol of dUMP was bound/mol of enzyme with a dissociation constant of 4×10^{-7} *M*. Other than the limited observations cited above, there have been

† From the Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201. *Received September 15, 1975.* Supported in part by a grant from the Institute of General Medical Sciences (GM-20371), PHS/DHEW.

[1] Abbreviations used are: H_4folate, 5,6,7,8-tetrahydrofolate; 5,10-CH_2H_4folate, 5,10-methylene tetrahydrofolate; H_2folate, 7,8-dihydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; 4*N*-OH-dCMP, N^4-hydroxydeoxycytidylate.

few definitive studies on the binding characteristics of thymidylate synthetase with substrates or substrate analogues. Such studies are critical to an understanding of the role of folate derivatives and of nucleotides in regulating enzyme activity and for an accurate assessment of the number and affinity of substrate binding sites. Since equilibrium dialysis is one of the most precise methods for obtaining information of this nature, it was employed in the present study to measure the binding of dUMP, dTMP, and FdUMP to thymidylate synthetase. In addition, it will be shown that the nature of the folate analogue and buffer used can greatly affect the binding characteristics of the enzyme for the nucleotide analogues.

Experimental Procedures

Materials

Thymidylate synthetase was purified and crystallized from methotrexate-resistant *L. casei* as described previously (Galivan et al., 1975). Enzyme solutions (15–20 mg/ml) were prepared by dissolving the crystalline protein in 50 m*M* potassium phosphate (pH 7.0) containing 20 m*M* 2-mercaptoethanol and then dialyzed against this buffer for 16 hr. The resulting preparation had a specific activity of 3.2–3.3 units/mg of protein when assayed spectrophotometrically (Galivan et al., 1974) in 50 m*M* potassium phosphate (pH 7.0). A unit is defined as the amount of enzyme required to produce 1 μmol of H_2folate/min at 30°. The molar concentration of enzyme was based on an A_{280} of 1.35 per mg of protein per ml and a molecular weight of 70 000 (Dunlap et al., 1971).

[2-^{14}C]Fluorodeoxyuridine obtained from Schwarz/Mann was converted to [2-^{14}C]FdUMP (ϵ 8.08 $\times$ 10^3 M^{-1} cm^{-1} at 265 nm, Mukherjee and Heidelberger, 1962) by a modification of the procedure of Yoshikawa et al. (1969) and purified by paper chromatography (ethanol–1 *M* ammonium acetate (pH 8.0), 7:3). Other [2-^{14}C]- and [6-^{3}H]deoxynucleoside 5′-monophosphates were purchased from Schwarz/Mann and purified by Dowex-1 formate column chromatography (Lorenson et al., 1967). The radioactive deoxynucleotides were diluted with carrier to a specific activity of 1–3 $\times$ 10^7 dpm/μmol. 4-*N*-OH-dCMP was prepared from dCMP as described earlier (Maley and Maley, 1964).

Folic acid, from Sigma Chemical Co., normally exhibited a single fluorescent spot on cellulose thin-layer chromatography (Polygram Cel$_{254}$, Macherey-Nagel & Co.) with 0.1 *M* potassium phosphate (pH 6.0) as developer. When impure samples were obtained they were purified by DEAE-cellulose column chromatography with a linear gradient of 0.1–0.8 *M* NH_4HCO_3, followed by ascending paper chromatography with *tert*-butyl alcohol–20% NH_4OH–H_2O (1:1:8). H_2folate (ϵ 28.4 $\times$ 10^3 M^{-1} cm^{-1} at 282 nm) was prepared from folic acid by reduction with sodium dithionite (Blakley, 1960a). H_4folate was prepared by the catalytic hydrogenation of folic acid according to Lorenson et al. (1967). The biologically active (+) isomer of 5,10-CH_2H_4folate was synthesized enzymically (Mathews and Huennekens, 1960) and purified by column chromatography on DEAE-cellulose with a linear gradient of 0.1–0.5 *M* NH_4HCO_3 containing 0.10 *M* 2-mercaptoethanol. The concentration of this folate, which was determined with thymidylate synthetase and an excess of dUMP, agreed with that obtained from the extinction coefficient (ϵ 32 $\times$ 10^3 M^{-1} cm^{-1} at 294 nm) (Blakley, 1960b). (−)5,10-CH_2H_4folate was synthesized enzymically by the technique of Kisliuk and Gaumont (1971) and purified by DEAE-cellulose column chromatography as described for the (+) isomer. Methotrexate was purchased from Lederle Laboratories and purified by the technique described above for folic acid as was [2-^{14}C]folic acid and [3′,5′-^{3}H]methotrexate (Amersham Searle Corp.). Radioactive folate coenzymes were synthesized as described above for the nonradioactive derivatives. The specific activities of folate and its analogues varied from 0.5 to 1 $\times$ 10^7 dpm/μmol.

Methods

Equilibrium Dialysis. The Kontron-Diapack apparatus described by Weder et al. (1971) was used in all of the studies. Regenerated cellulose membranes were boiled for 10 min in glass-distilled water, rinsed several times, and stored at 4° in the buffer to be used. The membranes were blotted to remove excess liquid prior to use. The protein and ligand samples (60 μl each) were added to opposite sides of the dialysis membrane with a Hamilton microsyringe. Protein concentrations were from 5 to 15 μM (0.35–1.05 mg/ml), radioactive ligands were from 0.002 to 0.6 m*M*, and 2-mercaptoethanol was included in all experiments at a concentration of 20 m*M*. The composition of the buffers and nonradioactive ligands employed is indicated in the Results. In all cases, equimolar amounts of each component were added to both sides of the membrane. After 4 hr of dialysis at 28°, the entire sample, amounting to 50–56 μl, was recovered from each chamber. Duplicate 20-μl samples were added to 10 ml of Aquasol and counted in a Beckman LS-250 scintillation counter. The duplicates agreed routinely within 2%. Controls, with thymidylate synthetase alone, revealed no loss in activity during dialysis. Dephosphorylation of the nucleotides could not be detected on incubation of the enzyme with FdUMP, dUMP, or dTMP for periods up to 20 hr at 28°.

The concentration of bound nucleotide was determined from the difference in radioactivity between the protein and ligand sides of the chamber, with radioactivity in the ligand side taken as a measure of the unbound or free ligand. No correction was made for Donnan effects. Because of the specific activity of the nucleotides and amounts of enzyme used, accurate dissociation constants below 10^{-8} *M* could not be determined.

Nucleotide binding data were calculated from the linearized equation of the law of mass action developed by Scatchard (1949):

$$\frac{\bar{\upsilon}}{[L_f]} = -\frac{1}{K_D}(\bar{\upsilon} - \eta) \qquad (1)$$

where $\bar{\upsilon}$ is the experimentally determined ligand bound in moles/mole of protein, $[L_f]$ is the free ligand concentration, K_D is the dissociation constant, and η is the extrapolated number of ligand binding sites/mole of protein. By plotting $\bar{\upsilon}/[L_f]$ vs. $\bar{\upsilon}$, the slope, $-1/K_D$, and abscissa intercept, η, are obtained. The $-1/K_D$ and η were determined from the experimental points by regression analysis. In those cases where nonlinear slopes were observed, first-order regression analysis was applied separately to each linear section of the curve. The curves were developed from a computer program with a RDP11/45 Digital Equipment Corporation computer.

Results and Discussion

Effect of Buffer on Deoxynucleotide Binding. Our initial studies are designed to assess the binding of dUMP to the

Table I: Binding of dUMP and dTMP to *L. casei* Thymidylate Synthetase.[a]

Folate Added	Buffer	Binding sites (η)	$K_D \times 10^6$
	dUMP		
None	Phosphate	1.18 ± 0.14	26.0 ± 4.3
None	Tris-HCl	1.16 ± 0.16	1.80 ± 0.25
None	Tris-HCl, $MgCl_2$	0.94 ± 0.08	7.00 ± 1.40
H_2 folate	Phosphate	0.96 ± 0.23	6.40 ± 1.80
	Tris-HCl	1.10 ± 0.09	0.52 ± 0.03
	Tris-HCl, $MgCl_2$	0.98 ± 0.09	0.52 ± 0.05
Methotrexate	Tris-HCl	1.10 ± 0.21	0.46 ± 0.09
(−)5,10-CH_2H_4 folate	Tris-HCl	0.90 ± 0.14	3.20 ± 0.54
	dTMP		
None	Tris-HCl	0.94 ± 0.08	5.75 ± 0.70
H_2 folate	Tris-HCl	1.00 ± 0.19	3.27 ± 0.12
(+)5,10-CH_2H_4 folate	Tris-HCl	0.83 ± 0.16	22.6 ± 3.37

[a] The equilibrium dialysis was carried out as described under Methods. The buffers used were 50 m*M* potassium phosphate (pH 7.0); 50 m*M* Tris-HCl (pH 7.1); and 50 m*M* Tris-HCl (pH 7.1) with 20 m*M* $MgCl_2$. Where folates were present, the following concentrations were used: H_2 folate, 0.3 m*M*; methotrexate, 0.2 m*M*; (−)5,10-CH_2H_4 folate, 0.15 m*M*; (+)5,10-CH_2H_4 folate, 0.1 m*M*. The concentrations and specific activities of [2-^{14}C] dUMP and [2-^{14}C] dTMP were varied as required for each experiment.

Table II: Binding of FdUMP to *L. casei* Thymidylate Synthetase.[a]

Folate Added	Buffer	Binding Sites (η)	$K_D \times 10^6$
		Single Site	
None	Phosphate	1.04 ± 0.07	97.0 ± 12
None	Phosphate[b]	1.00 ± 0.14	7.8 ± 1.4
Methotrexate	Phosphate	1.10 ± 0.13	10.3 ± 1.1
		Two Equivalent Sites	
None	Tris-HCl	2.20 ± 0.24	37.2 ± 3.6
None	Tris-HCl, $MgCl_2$	2.10 ± 0.25	21.5 ± 2.3
(+)5,10-CH_2H_4 folate	Phosphate	1.89 ± 0.13	<0.01
	Tris-HCl	1.75 ± 0.15	<0.01
(−)5,10-CH_2H_4 folate	Tris-HCl	1.52 ± 0.16	2.26 ± 0.17
Methotrexate	Tris-HCl	1.49 ± 0.13	2.20 ± 0.12

Folate Added	Buffer	Two Nonequivalent Sites: *A*	*B*	*A'*	*B'*
H_2 folate	Phosphate	1.42 ± 0.05	1.90 ± 0.16	1.2 ± 0.05	4.0 ± 1.6
	Tris-HCl	1.05[c]	1.85 ± 0.10	<0.01	2.7 ± 0.5
	Tris-HCl $MgCl_2$	1.05[c]	1.80 ± 0.11	<0.01	3.3 ± 0.4
(−)5,10-CH_2H_4-folate	Phosphate	0.93 ± 0.09	1.65 ± 0.33	1.3 ± 0.27	30 ± 16

[a] The equilibrium dialysis was carried out as described under Methods. The buffers used were: 50 m*M* potassium phosphate (pH 7.0); 50 m*M* Tris-HCl (pH 7.1); and 50 m*M* Tris-HCl (pH 7.1) with 20 m*M* $MgCl_2$. Where folates were present the following concentrations were used: H_2 folate, 0.3 m*M*; (+)5,10-CH_2H_4 folate, 0.03 m*M*; (−)5,10-CH_2H_4 folate, 0.1 m*M*; methotrexate, 0.2 m*M*. Where nonequivalent sites were observed, A is the first or high affinity site, and B the sum of the first and the second or low affinity site. A' and B' are the respective K_D's of A and the second binding site. [b] The concentration was 5 m*M* in this experiment. [c] Estimated from Figure 3B by dropping a perpendicular from the point where the lines intersect.

L. casei thymidylate synthetase and also to evaluate the effect of folates on this binding. The first part of the study which included about 15 experimental points ranging from a $\bar{\nu}$ of 0.2–1.1 indicates that the enzyme possesses a single binding site for dUMP with a K_D of 1.8×10^{-6} *M* when measured in 50 m*M* Tris-HCl (Table I). No evidence for a second site was apparent which is significant in terms of the FdUMP binding studies described below. A similar finding was obtained by Leary et al. (1975) using circular dichroism, but their reported K_D was 4×10^{-7} *M*. The fact that a direct measure of the free ligand concentration is not possible by the CD procedure may be responsible for this difference. The addition of 20 m*M* $MgCl_2$ to the Tris-HCl buffer system did not alter the number of binding sites but did increase the K_D by fourfold (Table I).

A more dramatic increase in the K_D for dUMP was obtained when the same experiment was conducted in 50 m*M* potassium phosphate (pH 7.0). In this instance, a single binding site was measured but with a K_D of 26×10^{-6} *M* (Table I). The product of the reaction, dTMP, also exhibited single site behavior but was less tightly bound than dUMP under comparable buffer conditions (Table I). The fact that dTMP binding could not be detected and that dUMP binding was impaired in potassium phosphate suggests that phosphate competes for the synthetase's substrate binding site with the deoxynucleoside 5′-monophosphates.

Effect of Folate Analogues on Deoxynucleotide Binding. The affinity of the enzyme for dUMP was markedly affected by the presence of H_2folate, but regardless of the folate analogue employed, the Scatchard plot was always linear and extrapolated to a single site for dUMP. As shown in Table I, H_2folate increased the affinity for dUMP approximately fourfold in phosphate buffer and 3.5-fold in Tris-HCl. Of interest is the finding that methotrexate also increased the affinity of the enzyme for dUMP approximately 3.5-fold, while (−)5,10-CH_2H_4folate decreased the affinity slightly. The binding of dTMP was not appreciably affected by H_2folate but was impaired by (+)5,10-CH_2H_4folate (Table I). From these data it can be concluded that native thymidylate synthetase binds 1 mol of dUMP or dTMP, and that the binding of dUMP is enhanced by folate analogues (Table I).

Binding of FdUMP. Previous studies using a variety of techniques (Aull et al., 1974b,c; Galivan et al., 1974, 1975; Santi et al., 1974) have shown that 2 mol of FdUMP are bound/mol of thymidylate synthetase in the presence of (±)5,10-CH_2H_4folate. Similar results have been obtained now by equilibrium dialysis where, with saturating amounts of (+)5,10-CH_2H_4folate, 1.89 mol of FdUMP was bound in 50 m*M* potassium phosphate buffer and 1.75 mol in 50 m*M* Tris-HCl (Table II). Due to the extremely high affinity of FdUMP in this complex as evidenced by the straight line parallel to the abscissa, the K_D could not be calculated (Figure 1B and 1D). However, by using rate constants of association and dissociation, Santi et al. (1974) have estimated a K_D of 5×10^{-11} for FdUMP in the ternary complex.

FdUMP exhibited more classical reversible binding, but as with dUMP and dTMP (Table I) the affinity of the synthetase for FdUMP was affected greatly by buffer conditions (Table II). Thus in 50 m*M* Tris-HCl (Figure 1C), 2 mol of FdMP was bound/mol of enzyme ($K_D = 37 \times 10^{-6}$

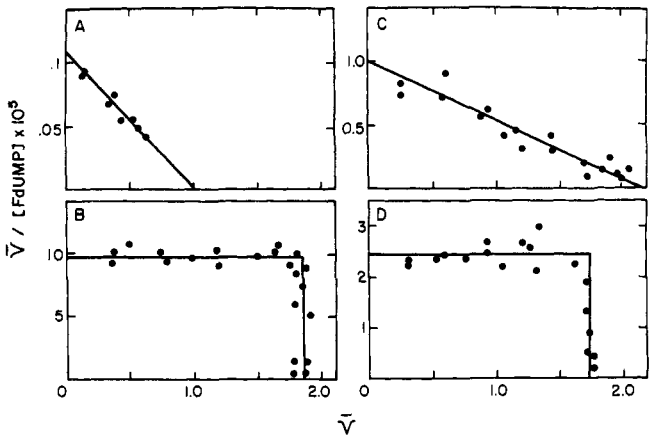

FIGURE 1: Binding of FdUMP to *L. casei* thymidylate synthetase in the presence and absence of (+)5,10-CH_2H_4folate. Equilibrium dialysis was conducted as described under Methods, with 50 m*M* potassium phosphate (pH 7.0) in (A) and (B) and 50 m*M* Tris-HCl (pH 7.1) in (C) and (D). (+)5,10-CH_2H_4folate at a final concentration of 0.03 m*M* was added to both sides of the membrane in (B) and (D); no additions were made in (A) and (C).

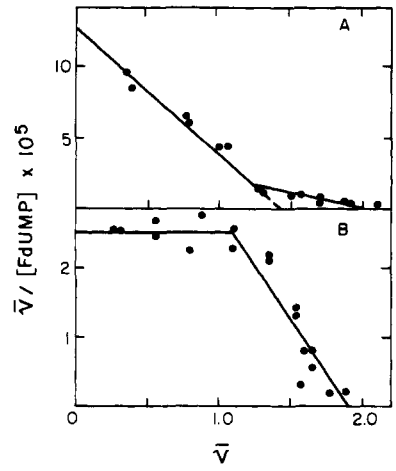

FIGURE 2: Binding of FdUMP to *L. casei* thymidylate synthetase in the presence of H_2folate. Equilibrium dialysis was conducted as described under Methods, with 0.3 m*M* H_2folate included in (A) which contained 50 m*M* potassium phosphate (pH 7.0); and in (B) which contained 50 m*M* Tris-HCl (pH 7.1).

M), but in 50 m*M* potassium phosphate (Figure 1A), only a single FdUMP site was evident ($K_D = 97 \times 10^{-6}$ *M*). It appears that the two binding sites for FdUMP are equivalent in Tris-HCl, but only one is available in phosphate buffer. The latter effect is probably due to the phosphate ion since potassium chloride did not alter the results shown in Figure 1C, whereas reduction of the potassium phosphate concentration to 5 m*M* increased the affinity by 12-fold ($K_D = 7.8 \times 10^{-6}$ *M*) as indicated in Table II. Despite this increase, the FdUMP binding was still restricted to one site.

When FdUMP binding was measured in the presence of H_2folate and 50 m*M* potassium phosphate, a second binding site became evident through a deviation from linearity in the Scatchard plot (Figure 2A), with the first site possessing a K_D of 1.2×10^{-6} *M* ($\eta = 1.4$) and the second a K_D of 4×10^{-6} *M* ($\eta = 1.0$). This experiment was repeated four times at 28° and twice at 0°, and in all cases the η value for the first site was 1.3–1.4. The reason for this deviation from unity is not understood but similar results were obtained with a nitrocellulose filter assay (Galivan et al., 1974; Santi et al., 1974) and by circular dichroism (CD) analysis (Galivan et al., 1975). The binding constant for each site was 80- and 24-fold lower in phosphate than for the single FdUMP site in the absence of H_2folate. A biphasic curve was also observed with H_2folate and 50 m*M* Tris-HCl as the buffer, but in this case both moles of FdUMP were bound more tightly (Figure 2B). The binding of the first mole was essentially stoichiometric with a K_D below 10^{-8} *M*, while the second site had a K_D of 2.7×10^{-6} *M*. Similar results were found with Tris-HCl supplemented with 20 m*M* $MgCl_2$. Since these biphasic curves could result from the contamination of H_2folate with H_4folate or 5,10-CH_2H_4folate, H_2folate was prepared by the technique of Blakley (1960a) and purified by DEAE-cellulose column chromatography. Neither H_4folate nor 5,10-CH_2H_4folate could be detected as contaminants of the H_2folate when assayed spectrally or enzymically. The experiments described in Figure 2 were then repeated with the purified H_2folate and results identical with those in the initial experiment were obtained. When (−)5,10-CH_2H_4folate was present in the potassium phosphate buffer system, a biphasic curve similar to that in Figure 2A and indicative of two classes of binding sites was observed; one with an η value of 0.93 and the other with an η of 1.65. With methotrexate in place of (−)5,10-CH_2H_4folate, only a single binding site could be shown despite a tenfold increase in its affinity (Table II). In contrast, the addition of either methotrexate or (−)5,10-CH_2H_4folate to the Tris-HCl buffer system decreased the number of equivalent binding sites to about 1.5 for FdUMP and lowered the K_D value in each system by 17-fold.

Competitive Binding between dUMP, dTMP, and FdUMP. Earlier kinetic and inhibition studies indicated that dUMP, dTMP, and FdUMP probably compete for the same binding site on thymidylate synthetase (Reyes and Heidelberger, 1965; Lorenson et al., 1967). It was of interest therefore to determine whether this relationship held for the *L. casei* synthetase. If competition of two nucleotides such as dUMP and dTMP for a single site is involved, the binding of each nucleotide is governed by the following equation (Englund et al., 1969):

$$\frac{\bar{\nu}_A}{[\text{deoxynucleotide}_A]} = -\frac{1}{K_A}\left(\frac{1}{\bar{\nu}_A} + \frac{1}{\bar{\nu}_B} - 1\right) \qquad (2)$$

By plotting $\bar{\nu}_{dUMP}/[dUMP_f]$ against ($\bar{\nu}_{dUMP} + \bar{\nu}_{dTMP}$) and $\bar{\nu}_{dTMP}/[dTMP_f]$ against ($\bar{\nu}_{dUMP} + \bar{\nu}_{dTMP}$) straight lines with a common intercept of 1 should be obtained, providing the two nucleotides bind to the same site. The symbols used are similar to those described for eq 1, but in this case A and B are separate ligands and f represents the concentration of unbound or free ligand. When this plot was constructed from an experiment using [6-^{3}H]dUMP and [2-^{14}C]dTMP (Figure 3A), it was found that dUMP and dTMP do indeed share a common site. The dissociation constants calculated using the competition data in Figure 3 agreed with those obtained when each nucleotide was measured separately (Table I).

A similar experiment was conducted with [2-^{14}C]FdUMP replacing [2-^{14}C]dTMP but with a low postassium phosphate buffer concentration (5 m*M*). The latter concentration restricts FdUMP binding to a single site of relatively high affinity, enabling competition for this site to be measured with an acceptable degree of accuracy. The results of this experiment (Figure 3B) were similar to those for Figure 3A, with dUMP possessing a greater affinity for the binding site than FdUMP. This result substantiates the

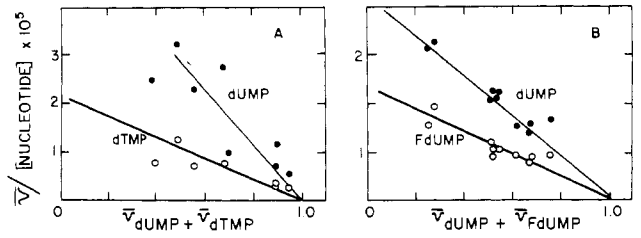

FIGURE 3: Competition between dUMP, dTMP, and FdUMP for binding to *L. casei* thymidylate synthetase as determined by a Scatchard analysis. Equilibrium dialysis was conducted as described under Methods. Varying amounts of [6-^{3}H]dUMP and [2-^{14}C]dTMP were added to the ligand side of the membrane in (A). All samples contained 50 m*M* Tris-HCl (pH 7.1). The data were calculated and plotted according to Englund et al. (1969), whereby the fractional binding $\bar{v}_A$ of a given ligand A is divided by its free concentration [A_f] and then plotted against the sum of the fractional binding of both ligands ($\bar{v}_A + \bar{v}_B$). The same protocol was used in (B) as in (A), except for replacement of [2-^{14}C]dTMP by [2-^{14}C]FdUMP, and Tris-HCl by 5 m*M* potassium phosphate (pH 7.0).

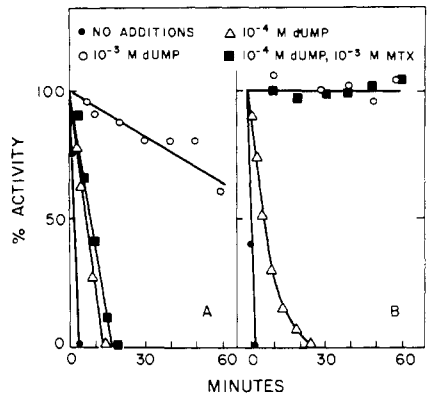

FIGURE 4: Effect of temperature on the stability of *L.* casei thymidylate synthetase. Thymidylate synthetase (4 μM) was incubated at 55° in 0.35-ml solutions of (A) 50 m*M* potassium phosphate (pH 7.0) plus 50 m*M* 2-mercaptoethanol or (B) 50 m*M* Tris-HCl (pH 7.0) plus 50 m*M* 2-mercaptoethanol, and the indicated additions; 10-μl aliquots were removed at the indicated times and assayed spectrally (Galivan et al., 1974). MTX, methotrexate.

tighter binding of dUMP relative to FdUMP when measured independently in the absence of folates (Table I vs. Table II).

Evidence for Binding through the Use of Heat Stability Studies. Various combinations of substrates have been reported to stabilize thymidylate synthetase in vitro (Bonney and Maley, 1975; Lorenson et al., 1967; Roberts and Loehr, 1971) and as a result have been postulated to play a role in modulating synthetase levels in vivo. From the data in Table II it might be predicted that a more stable ternary complex of methotrexate-enzyme-dUMP exists in Tris-HCl than in potassium phosphate. This prediction was tested by heat stability studies in the two buffer systems. As shown in Figure 4, enzyme alone in the presence of either buffer loses all of its activity in 5 min at 55°. However, partial stabilization could be effected by 10^{-3} *M* dUMP in potassium phosphate buffer (Figure 4A) and complete stabilization by 10^{-3} *M* dUMP in Tris-HCl (Figure 4B). The latter result reflects the 15-fold greater affinity of the synthetase for dUMP in Tris-HCl (Table I). When heat inactivation was conducted in the presence of a combination of 10^{-4} *M* dUMP and 10^{-3} *M* methotrexate and Tris-HCl, a dramatic synergistic effect was obtained with no loss in enzyme activity noted after 60 min at 55°. In contrast complete inactivation was obtained after 15 min in phosphate buffer. Methotrexate alone in either buffer system at 10^{-3} *M* did not protect the synthetase.

Table III: Binding of Folate Analogues to Thymidylate Synthetase.[a]

Folate Analogue	Deoxynucleotide Added	Binding Sites (η)	$K_D \times 10^6$
Folic acid	None	ndb[b]	
	dUMP	0.82 ± 0.31	21.0 ± 14
Methotrexate	None	ndb	
	dUMP	1.03 ± 0.29	24.5 ± 12
H_2 folate	None	ndb	
	dUMP	0.83 ± 0.25	13.2 ± 5.9
	4-*N*-OH-dCMP	1.03 ± 0.07	1.60 ± 0.01
(+)5,10-CH_2H_4folate	None	ndb	
	4-*N*-OH-dCMP	1.1 ± 0.10	1.90 ± 0.02

[a] The equilibrium dialysis was carried out as described under Methods. The buffer used was 50 m*M* Tris-HCl (pH 7.1), with 20 m*M* $MgCl_2$. Deoxynucleotides, where included, were added to both sides of the membrane at a concentration of 0.17 m*M*. [3′,5′-^{3}H]-Methotrexate and [2-^{14}C] folate derivatives were employed as radioactive ligands. [b] ndb, no detectable binding; as determined by the difference in radioactivity between the protein and ligand side of the membrane at folate analogue concentrations from 0.005 to 0.5 m*M*.

These effects are analogous to those obtained with this enzyme in extracts of rat liver cells (Bonney and Maley, 1975) where it was proposed that the observed elevation in thymidylate synthetase activity is in part a consequence of the binding of methotrexate or the more effective combination of methotrexate and dUMP. Based on the results presented in Figure 4 and Table III, ternary complex formation may also be important in stabilizing the *L. casei* synthetase.

Binding of Folate Analogues. Since thymidylate synthetase did not appear to be stabilized by methotrexate alone, it was of interest to determine if this and other folate derivatives were bound by the enzyme. As shown in Table III, evidence for the binding of (+)5,10-CH_2H_4folate, H_2folate, folate, or methotrexate could not be obtained at concentrations up to 0.5 m*M*, in the absence of deoxynucleotides. However, single-site binding for each of these folate derivatives was readily apparent in the presence of dUMP or 4-*N*-OH-dCMP. The latter nucleotide, a competitive inhibitor of the *L. casei* enzyme with respect to dUMP, $K_I = 8 \times 10^{-6}$ *M* (unpublished results, J. H. Galivan), also increased the affinity of the enzyme for [2-^{14}C]H_2folate by eightfold when compared with dUMP. A Scatchard plot measuring binding of (+)5,10-CH_2H_4 [2-^{14}C]folate in the presence of 4-*N*-OH-dCMP was linear and extrapolated to a single binding site with a K_D of 1.9×10^{-6} *M*. In a similar experiment with FdUMP in place of 4-*N*-OH-dCMP, 1.8 binding sites were found for (+)5,10-CH_2H_4[2-^{14}C]folate but the affinity was too high to be measured accurately. In contrast, the binding of (+)5,10-CH_2H_4[2-^{14}C]folate could not be detected in the presence of dTMP.

Although the binding of the (−)5,10-CH_2H_4folate diastereoisomer was not measured, it is expected that similar results to those with the (+) diastereoisomer would be obtained, except for the K_D which would probably be higher. This assumption is based on the finding that (−)5,10-CH_2H_4folate is an inhibitor of the synthetase (Kisliuk et al., 1974; Leary et al., 1974) and on CD studies with this compound (Galivan et al., 1975).

A comparison of the binding of folates and deoxyribonucleotides reveals that the latter are bound more tightly in the ternary complex than the former (Tables I and III). As indicated, the affinity of the synthetase was considerably greater for dUMP than for methotrexate or H_2folate in an incubation mixture consisting of the respective folate analogue, enzyme, dUMP, and Tris-HCl, $MgCl_2$.

Conclusions

In this study thymidylate synthetase has been found to possess only one binding site for dUMP under a variety of conditions, and although the binding is tighter in the presence of certain folate analogues, a second site has not been observed. A similar result was recently reported for dUMP and enzyme through the use of circular dichroism (Leary et al., 1975). These findings contrast with the fact that the enzyme consists of two apparently identical subunits (Loeble and Dunlap, 1972) and is inhibited completely by 2 equiv of FdUMP (Santi and McHenry, 1972; Santi et al., 1974; Galivan et al., 1974). Although the FdUMP inhibition data appear to support the concept of two active sites, several lines of evidence argue against it. The results of the dUMP, dTMP binding studies and the complete inhibition of the synthetase on reacting one of the enzyme's four sulfhydryl groups with a sulfhydryl reagent (Dunlap et al., 1971; Leary et al., 1975), an effect protected against by substrate (Leary et al., 1975), provide evidence in support of a single active site. The restriction of folate binding to a single site is also in concert with this concept. In addition, Aull et al. (1974a) have demonstrated that the synthetase is completely inactivated by the release of 1 mol of carboxy-terminal valine/mol of enzyme. The single active site thesis must be taken with some caution, however, since it is not possible to measure dUMP binding in the presence of (+)5,10-CH_2H_4folate due to its rapid conversion to dTMP. Despite this reservation, a clear distinction between the binding of the substrate, dUMP, and the inhibitor, FdUMP, becomes apparent when the binding of the two nucleotides is compared in 50 m*M* Tris-HCl (pH 7.1) (Table I and Figure 1C). It is evident from these results that, although the K_D of dUMP is roughly 20-fold lower than that for FdUMP, there is only one binding site for dUMP, and two for FdUMP. Binding of the second mole could be prevented by conducting the experiments in potassium phosphate buffer.

When FdUMP binding was measured in the presence of various folate derivatives, the extent of reaction was influenced by the particular folate and also by the buffer system employed. In every case examined, the folate derivative increased the affinity of FdUMP binding, and, in all but one instance (methotrexate in phosphate buffer), the extent of binding approached 2 mol of FdUMP/mol of enzyme. Equivalent two-site binding was obtained with (−)5,10-CH_2H_4folate or methotrexate in Tris-HCl buffer, while nonequivalent two site binding, where the second mole of FdUMP is bound less tightly than the first, was obtained with (−)5,10-CH_2H_4folate or H_2folate in phosphate buffer. Nonequivalent binding was also obtained with H_2folate in Tris-HCl. Because of the high affinity of FdUMP binding in the presence of (+)5,10-CH_2H_4folate with either buffer system, it was not possible to determine whether the affinity for FdUMP at the two sites is identical. Additional evidence that the two FdUMP sites may not be identical has come from studies in our laboratory which indicate that the FdUMP at one site is sensitive to urea (Galivan et al., 1974). Danenberg et al. (1974) have also reported that a species of the ternary complex can be isolated which contains only 1 mol of FdUMP.

It should be emphasized that the binding of the folate analogues differs significantly from that of the deoxynucleotides in that the folates, at concentrations as high as 0.5 m*M*, do not bind to the enzyme, in the absence of deoxynucleotides. These results agree with our earlier observations (Galivan et al., 1975), where ternary complex formation with synthetase, folate, and FdUMP was detected by CD measurements, but no change in the spectrum was evident with just enzyme and folate.

Several investigations have been conducted to determine if the substrates interact with thymidylate synthetase by a random or an ordered pathway, with one substrate bound obligatorily, prior to the other. Blakley (1963) using the synthetase from *Streptococcus faecalis* and Reyes and Heidelberger (1965) using enzyme from Ehrlich ascites cells have proposed an ordered mechanism in which 5,10-CH_2H_4folate binds first, followed by dUMP. In contrast to this view, the lack of binding of folate derivatives in the absence of the appropriate deoxynucleoside 5′-monophosphate lends support to a mechanism of ordered addition in which dUMP binds before (+)5,10-CH_2H_4folate, as suggested previously from kinetic and heat stability studies with the chick embryo thymidylate synthetase (Lorenson et al., 1967).

It should be emphasized that none of the binding studies were conducted with the natural or folylpolyglutamates which might yield somewhat different results from those obtained in this study with the monoglutamates. The striking inhibition of thymidylate synthetase reported by Kisliuk et al. (1974) for the folylpolyglutamates, relative to the monoglutamates, suggests such a possibility.

Acknowledgments

The authors gratefully acknowledge the assistance of Dr. Stephen Kim in constructing the computer program for the regression analysis and also the excellent technical assistance of Zenia Nimec and Thaisa Beach.

References

Aull, J. L., Loeble, R. B., and Dunlap, R. B. (1974a), *J. Biol. Chem. 249,* 1167.

Aull, J. L., Lyon, J. A., and Dunlap, R. B. (1974b), *Microchem. J. 19,* 210.

Aull, J. L., Lyon, J. A., and Dunlap, R. B. (1974c), *Arch. Biochem. Biophys. 165,* 805.

Blakley, R. L. (1960a), *Nature (London) 188,* 231.

Blakley, R. L. (1960b), *Biochem. J. 74,* 71.

Blakley, R. L. (1963), *J. Biol. Chem. 238,* 2113.

Bonney, R. J., and Maley, F. (1975), *Cancer Res. 35,* 1950.

Danenberg, P. V., Langenbach, R. J., and Heidelberger, C. (1974), *Biochemistry 13,* 926.

Dunlap, R. B., Harding, N. G. L., and Huennekens, F. M. (1971), *Biochemistry 10,* 88.

Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A. (1969), *J. Biol. Chem. 244,* 3038.

Galivan, J. H., Maley, G. F., and Maley, F. (1974), *Biochemistry 13,* 2282.

Galivan, J. H., Maley, G. F., and Maley, F. (1975), *Biochemistry 14,* 3338.

Kisliuk, R. L., and Gaumont, Y. (1971), *Ann. N.Y. Acad. Sci. 186,* 438.

Langenbach, R. J., Danenberg, P. V., and Heidelberger, C.

(1972), *Biochem. Biophys. Res. Commun. 48,* 1565.
Leary, R. P., Beaudette, N., and Kisliuk, R. L. (1975), *J. Biol. Chem. 250,* 4864.
Leary, R. P., and Gaumont, Y. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 591.
Leary, R. P., and Kisliuk, R. L. (1971), *Prep. Biochem. 1,* 47.
Loeble, R. B., and Dunlap, R. B. (1972), *Biochem. Biophys. Res. Commun. 49,* 1671.
Lorenson, M. Y., Maley, G. F., and Maley, F. (1967), *J. Biol. Chem. 242,* 3332.
Maley, G. F., and Maley, F. (1964), *J. Biol. Chem. 239,* 1168.
Mathews, C. K., and Huennekens, F. M. (1960), *J. Biol. Chem. 235,* 3304.
McHenry, C. S., and Santi, D. V. (1974), *Biochem. Biophys. Res. Commun. 57,* 204.
Mukherjee, K. L., and Heidelberger, C. (1962), *Cancer Res. 22,* 815.
Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol. 1,* 14.
Roberts, D., and Loehr, E. V. (1971), *Cancer Res. 31,* 1181.
Santi, D. V., and McHenry, C. S. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1855.
Santi, D. V., McHenry, C. S., and Sommer, H. (1974), *Biochemistry 13,* 471.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Weder, H. G., Schildknecht, J., and Kesselring, P. (1971), *Am. Lab. 3,* 17.
Yoshikawa, M., Kato, T., and Takenishi, T. (1969), *Bull, Chem. Soc. Jpn. 42,* 3505.

The Mechanism of Quinonediimine Acceptor Activity in Photosynthetic Electron Transport†

James A. Guikema and Charles F. Yocum*

ABSTRACT: The rates of electron flow catalyzed by a variety of unsubstituted and C- or N-methylated quinonediimine electron acceptors in a reaction requiring photosystem II in KCN-inhibited chloroplasts vary according to the structure of acceptor used. Quinonediimine, but not quinone, electron acceptor activities are inhibited by a variety of uncouplers. Kinetic analysis of this inhibition shows that it is competitive. Low concentrations of aniline also inhibit the activity of C-methylated quinonediimines, but this appears to be due to a chemical reaction between the acceptor and aniline at low pH inside the chloroplast. Light-induced uptake of a quinonediimine, *p*-phenylenediimine, was shown to occur in a DCMU-sensitive reaction. Methylamine uncoupling inhibits this uptake to the same extent as it inhibits electron flow. Experiments with a lipophobic acceptor, *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine, indicate that it catalyzes electron flow by the same mechanism as other quinonediimines. A model is proposed to account for quinonediimine-catalyzed electron flow.

Saha et al. (1971) have shown that oxidized quinones and oxidized *p*-diaminobenzene compounds (quinonediimines) are effective mediators of electron transport in spinach chloroplasts, and that the efficiency of photophosphorylation obtained with these compounds is approximately half that obtained with electron acceptors such as ferricyanide or methyl viologen. Inhibition of electron transport by treatment of chloroplasts with KCN, which inactivates plastocyanin (Ouitrakul and Izawa, 1973), or by the plastoquinone antagonist DBMIB[1] (Trebst and Reimer, 1973a,b; Izawa et al., 1973), have relatively little effect on quinonediimine photoreduction. These observations implicate the reducing side of photosystem II as the site of electron donation to these compounds. Saha et al. (1971) and Trebst (1974) have proposed models for the electron acceptor activity of quinones and quinonediimines in which the oxidized species of the acceptor molecule, a lipophile, penetrates a membrane (outside to inside), undergoes photoreduction, and then transfers reducing equivalents back to the external medium by a repenetration of the membrane from inside to outside. The penetration of the membrane in these models is ascribed to the lipophilic nature of the acceptor and a shuttle of the acceptor molecules in the light.

Although addition of adenosine diphosphate and inorganic phosphate as substrates for photophosphorylation does not markedly enhance the rate of photoreduction of either quinones or quinonediimines, uncouplers have been shown to affect this pathway of electron flow. Gould and Ort (1973) showed that oxygen evolution in the presence of DBMIB, a reaction which required a quinonediimine acceptor (PDox), was sensitive to the uncoupler methylamine. This effect was attributed to inhibition of photosystem II activity by the uncoupler. Trebst and Reimer (1973a) ob-

† From the Department of Cell and Molecular Biology, The University of Michigan, Ann Arbor, Michigan 48104. *Received March 27, 1975.* This investigation was supported in part by a research grant from the H. H. Rackham School of Graduate Studies of the University of Michigan.

[1] Abbreviations used are: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; PDox, *p*-phenylenediimine; DADox, 2,3,5,6-tetramethyl-*p*-phenylenediimine; DMPDox, *N,N*-dimethyl-*p*-phenylenediimine; DATox, 2,5-diiminotoluene; TMPDox, *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine; DMQ, 2,5-dimethyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; *m*-Cl-CCP, *m*-chlorocarbonylcyanide phenylhydrazone; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid.