Effect of 5,10-Methylenetetrahydrofolate on the Dissociation of 5-Fluoro-2′-deoxyuridylate from Thymidylate Synthetase: Evidence for an Ordered Mechanism†

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ABSTRACT: A study of the kinetics of breakdown of the ternary covalent complex among *Lactobacillus casei* thymidylate synthetase, 5-fluoro-2′-deoxyuridylate (FdUMP), and 5,10-methylenetetrahydrofolate (5,10-CH_2H_4 folate) is described. Formation of the complex is rapid ($k_{app} = 2.0 \times 10^8$ M^{-1} min^{-1} at 37 °C) with concomitant enzyme inactivation, but a slow enzyme-catalyzed reversal of the complex also occurs. If the complex is incubated with excess 2′-deoxyuridylate (dUMP), enzyme activity is recovered with a half-time ($t_{1/2}$) value of 36 min at 37 °C. The rate of recovery, however, is not influenced by changing the dUMP concentration, which indicates a first-order dissociation of FdUMP from the complex. The dissociation of [^{3}H]FdUMP from the complex, as measured by its rate of exchange with unlabeled FdUMP, proceeds at the same rate as recovery of enzyme activity in the absence of added 5,10-CH_2H_4folate but is suppressed as levels of 5,10-CH_2H_4folate are increased. The reciprocal of the first-order rate constant ($t_{1/2}$) for dissociation of FdUMP is a linear function of 5,10-CH_2H_4folate concentration, increasing from 36 min at zero cofactor to 840 min at 3 mM cofactor. Conversely, the dissociation of labeled cofactor is entirely unaffected by increased FdUMP concentrations. In the presence of the competitive inhibitor 5-bromo-2′-deoxyuridylate (BrdUMP), substrate inhibition of the enzymatic reaction by 5,10-CH_2H_4folate was observed. This induced substrate inhibition is linear with 5,10-CH_2H_4folate concentration and competitive with respect to the substrate dUMP. The cofactor alone also gives substrate inhibition, but at much higher concentration than required with BrdUMP. 10-Methyltetrahydrofolate (10-MeH_4folate), an inhibitor competitive with 5,10-CH_2H_4folate, gives linear uncompetitive kinetics against dUMP. These data demonstrate a sequential ordered mechanism both for the enzymatic reaction and ternary complex formation in which binding of the nucleotide is a prerequisite to binding of 5,10-CH_2H_4 folate.

Thymidylate synthetase (EC 2.1.1.45) is a key enzyme in DNA biosynthesis, since it constitutes the only de novo source of dTMP[1] in most types of cells. The enzyme catalyzes a unique two-step reaction which involves sequential transfer of a one-carbon fragment from the cofactor, 5,10-CH_2H_4-folate, to dUMP (eq 1).

$$\text{dUMP} + \text{5,10-CH}_2\text{H}_4\text{folate} \rightarrow \text{dTMP} + \text{H}_2\text{folate} \quad (1)$$

There are a number of facets of the mechanism of this reaction that have not yet been completely elucidated.

Thymidylate synthetase is considered to be the primary target enzyme for the widely used antitumor agent 5-FU (Cohen et al., 1958; Danneberg et al., 1958). The active form of the drug, FdUMP, has long been known to be a very potent inhibitor of the enzyme (Cohen et al., 1958; Hartmann & Heidelberger, 1961), but only relatively recent investigations have elucidated the mode of interaction of FdUMP and thymidylate synthetase. All lines of evidence obtained so far are consistent with the idea that FdUMP acts as a quasi-substrate or K_{cat} inhibitor (Rando, 1974) which proceeds only part-way through the reaction sequence. As a result, a ternary covalent complex of enzyme, FdUMP, and cofactor is formed which is probably analogous in structure to a steady-state intermediate of the enzymatic reaction (Danenberg et al., 1974). The covalent bonds that link FdUMP and 5,10-CH_2H_4folate to the protein are chemically stable since they survive rigorous denaturing conditions involving acid precipitation and sodium dodecyl sulfate treatment (Langenbach et al., 1972). Under these conditions binding of FdUMP and cofactor to the protein is irreversible. However, if no denaturants have been added, the complex will slowly dissociate upon extensive dialysis with partial reactivation of the enzyme (Reyes & Heidelberger, 1965) and release of FdUMP (Langenbach et al., 1972). Santi et al. (1974) showed that the components of the complex undergo association and dissociation events as if there were no irreversible covalent bonds, since radiolabeled FdUMP in the complex could be readily exchanged with unlabeled FdUMP. The reverse reaction, therefore, involves enzyme-mediated cleavage of the covalent bonds between FdUMP, cofactor, and the protein, but due to the stability of these bonds the equilibrium greatly favors complex formation.

The objective of the present work was to obtain information about the enzymatic mechanism by a study of the dissociation of FdUMP from the ternary complex using competitive ligand binding techniques in conjunction with kinetics studies of the forward reaction. The effects of 5,10-CH_2H_4folate on FdUMP dissociation rates demonstrate an ordered mechanism for formation of the ternary complex which requires that the binding of FdUMP precede that of the cofactor, 5,10-CH_2H_4folate. Enzyme kinetics studies are described which are also in accord with a sequential ordered model of substrate binding.

Materials and Methods

Thymidylate synthetase was (sp act. 3.5 μmol/(min mg)) obtained from methotrexate-resistant *L. casei* as previously described (Danenberg et al., 1974). [^{3}H]FdUMP was syn-

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[1] Abbreviations used: dTMP, 2′-deoxythymidylic acid; dUMP, 2′-deoxyuridylic acid; FdUMP, 5-fluoro-2′-deoxyuridylic acid; BrdUMP, 5-bromo-2′-deoxyuridylic acid; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolate, also referred to as the cofactor; 5-FU, 5-fluorouracil.

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thesized from [6-^{3}H]FdUrd (0.4 Ci/mmol, Amersham/Searle) by a chemical phosphorylation as described previously (Danenberg & Heidelberger, 1976). BrdUMP was obtained from Sigma Chemical Co. Schleicher and Schuell nitrocellulose filters (BA85) were used for the filter assays. Dihydrofolate reductase was isolated from methotrexate-resistant *L. casei* and furnished to us by Dr. Richard Moran.

1,L-H_4folate was prepared enzymatically by reduction of H_2folate (Blakley, 1960) with dihydrofolate reductase in the presence of excess NADPH. When the reaction was complete, the mixture was placed on a DEAE-cellulose column and H_4folate eluted with a linear gradient of ammonium acetate (0–0.5 M) containing 1% β-mercaptoethanol. From the elution profile of this column, the H_4folate obtained appeared to be >98% pure and was stored as a lyophilized powder in glass ampoules sealed under vacuum. 10-MeH_4folate was prepared by a similar procedure involving dihydrofolate reductase reduction of 10-Mefolate and then purified by DEAE-cellulose chromatography. [^{14}C]5,10-CH_2H_4folate was synthesized from [2-^{14}C]folate (58 mCi/mmol, Amersham/Searle) by an identical procedure. 1,L-H_4folate was converted to 1,L-5,10-CH_2H_4folate by incubation with 30 mM formaldehyde for 30 min.

Assays for thymidylate synthetase activity were carried out as previously described (Danenberg et al., 1974). Initial velocities were measured in all cases.

Isolation of the Thymidylate Synthetase–FdUMP–5,10-CH_2H_4folate Complex. Thymidylate synthetase was incubated for 15 min with 0.01 mM FdUMP and 0.1 mM 5,10-CH_2H_4folate, with one or the other being radiolabeled as required by the experiment, in 1 mL of 50 mM Tris containing 50 mM β-mercaptoethanol and 50 mM KCl. A slurry of charcoal (10% by weight in water), containing 3% bovine serum albumin and 0.1% dextran, was added (0.3 mL). The suspension was vortexed for 30 s and then centrifuged for 15 min at a setting of 7 on an IEC table-top clinical centrifuge. The supernatant, which contained the enzyme–FdUMP–cofactor complex free of unbound ligands, was carefully removed with a Pasteur pipet. A control experiment with active enzyme showed that the charcoal treatment had no deleterious effects on enzyme activity. The completeness of the charcoal adsorption was checked by filtration of the supernatant through nitrocellulose filters as described in the next section. It was found that 95–100% of the radioactivity was filterable (protein bound).

Assay for Ligand Binding to Thymidylate Synthetase. A slight modification of the nitrocellulose filter method developed by Yarus & Berg (1970) and Santi et al. (1974a) was used to measure binding of radioactive ligands to thymidylate synthetase. After filtration of an aliquot of the reaction mixture containing no more than 50 μg of protein, filter discs were washed with 3 × 1 mL of 50 mM Tris buffer, pH 7.2, to remove unbound molecules. The discs were then placed in a counting vial and dissolved in 1 mL of ethylene glycol monomethyl ether. For liquid scintillation counting, 9 mL of RIA-Solve II (Research Products International, Elk Grove Village, Ill.) was added to each vial. The filtration efficiency was determined to be 40%, a value which was constant for the lot of filters used in these studies.

Data Processing. Reciprocal velocities were plotted graphically against the reciprocals of substrate concentrations.

Data from reciprocal plots were fitted to the appropriate rate equation by the least-squares method assuming equal variance for the velocities. The data analysis was carried out by Dr. W. W. Cleland, Department of Biochemistry, University of Wisconsin, using previously published Fortran programs (Cleland, 1967) for competitive patterns (eq 2) and a program written for a pattern involving substrate inhibition (eq 3) (W. W. Cleland, unpublished results).

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (2)$$

$$v = \frac{VA}{K_a + A + A^2/K_I} \quad (3)$$

Rate constants relevant to the proposed mechanism were determined by computer-fitting the data to eq 7 (see Results) rearranged to the forms of eq 2 or 3, depending on which substrate was varied. The terms in the above equations and in eq 7 have been defined by Cleland (1963a–c).

Results

The Effect of dUMP on the Recovery of Enzyme Activity from the Covalent Ternary Complex. In view of reports (Myers et al., 1975) which seemed to indicate that elevation of dUMP levels in FU-treated tumor cells could effectively overcome the FdUMP block of thymidylate synthetase and allow the tissue to recover, we were interested in determining whether dUMP could interact directly with the enzyme to influence the rate of enzyme reactivation from the ternary complex. The thymidylate synthetase–FdUMP–5,10-CH_2H_4folate complex freed of excess unbound ligands by charcoal treatment as described in Materials and Methods was incubated with dUMP at various concentrations, and the enzyme activity of aliquots was measured at intervals. It was found that recovery of enzyme activity, which occurred with $t_{1/2}$ of 36 min at 37 °C, was independent of dUMP concentrations over a range of 0.01 to 1 mM. Furthermore, the full original activity was eventually regained, demonstrating the ability of the enzyme to completely reverse the formation of the covalent bonds in the ternary complex formed with FdUMP and cofactor. In the absence of any added dUMP, however, the ternary complex showed no net increase in enzyme activity over all time periods measured. These observations suggest that enzyme activity is regained by a first-order dissociation of FdUMP followed by competitive binding of dUMP in the active site(s) vacated by FdUMP. If this is the case, exchange of [^{3}H]FdUMP from the ternary complex with unlabeled FdUMP, as measured by the filter binding technique, should proceed at the same rate as recovery of enzyme activity as determined by measuring the reaction rate, and, in fact, when the enzyme–[^{3}H]FdUMP–5,10-CH_2H_4folate complex was incubated with 0.1 mM unlabeled FdUMP to ensure sufficient isotope dilution of dissociated [^{3}H]FdUMP, the loss of tritium from the protein occurred with the same $t_{1/2}$ of 36 min as was determined for enzyme reaction.

The Effect of dUMP on the Tight Binding of FdUMP. It has not yet been established whether thymidylate synthetase has one catalytically functional site, two identical sites, or two nonidentical sites. The literature on this question is confusing: it has been shown repeatedly that FdUMP has two high-affinity binding sites per enzyme molecule (Sharma & Kisliuk 1973; Santi et al., 1974b; Galivan et al., 1975; Aull et al., 1974), whereas both equilibrium dialysis studies (Galivan et al., 1976) and circular dichroism data (Leary et al., 1975) have indicated one binding site for dUMP. Chemical modification studies are also conflicting. Plese & Dunlap (1977) found that maximum inhibition of enzyme activity was achieved with 1.6 mol of pCMB incorporated per enzyme molecule, while Galivan et al. (1977) observed that alkylation of only 1 cysteine residue per enzyme with a variety of agents produced complete

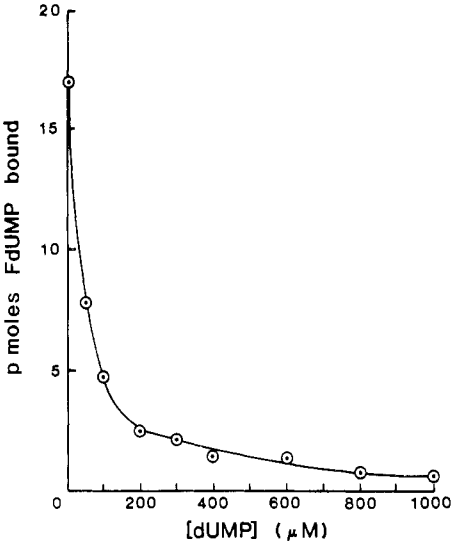


FIGURE 1: Competition of dUMP and FdUMP for binding sites on thymidylate synthetase. The binding of [^{3}H]FdUMP was measured at various concentrations of dUMP. Enzyme (0.1 μM) was added to a mixture of [^{3}H]FdUMP (1.0 μM), 5,10-CH_2H_4folate (100 μM), and dUMP at the concentration indicated in a total volume of 0.2 mL of Tris buffer (50 mM), pH 7.2. The mixture was incubated for 10 s and then filtered through a nitrocellulose filter to determine protein-bound [^{3}H]-FdUMP.

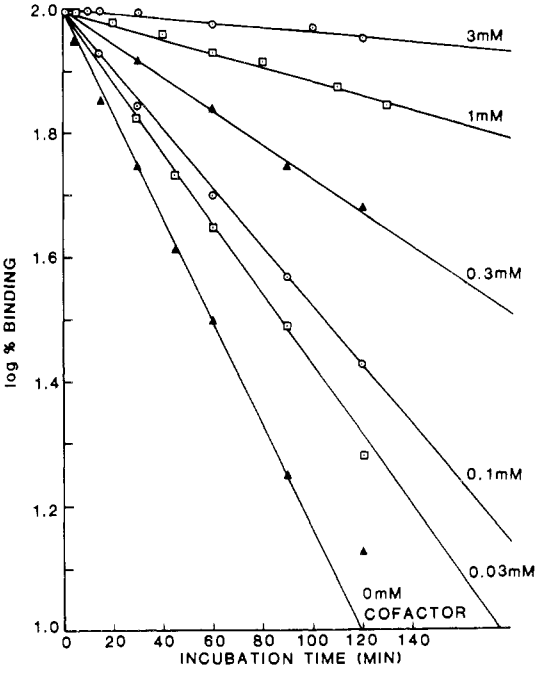


FIGURE 2: Loss of [^{3}H]FdUMP from the thymidylate synthetase-[^{3}H]FdUMP-5,10-CH_2H_4folate complex in the presence of 0.1 mM FdUMP as a function of 5,10-CH_2H_4folate concentration. The numbers on the various lines represent the concentration of 5,10-CH_2H_4folate. Aliquots were taken at indicated time intervals and assayed for protein-bound [^{3}H]FdUMP by the nitrocellulose filter method.

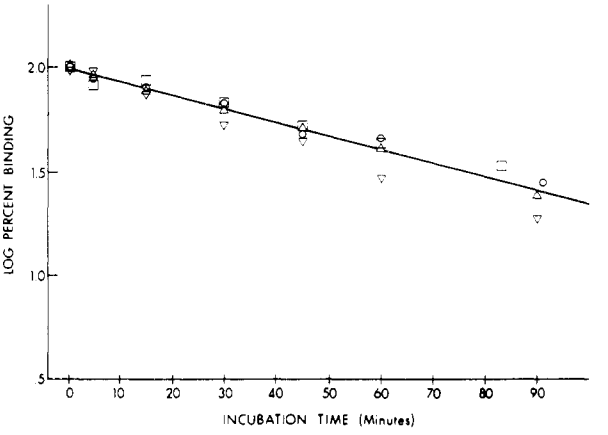


FIGURE 3: Loss of [^{14}C]-5,10-CH_2H_4folate from the thymidylate synthetase-FdUMP-[^{14}C]CH_2H_4folate complex as a function of FdUMP concentration: (○) 0.01 mM 5,10-CH_2H_4folate, no FdUMP; (□) 0.1 mM 5,10-CH_2H_4folate, no FdUMP; (△) 0.1 mM 5,10-CH_2H_4folate, 0.016 mM FdUMP; (▽) 0.1 mM 5,10-CH_2H_4folate, 0.160 mM FdUMP.

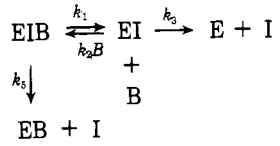
inactivation. The fact that full enzyme activity is regained from the initially inactive enzyme-FdUMP-cofactor complex suggests two alternatives which we can distinguish using the ligand binding to techniques described: (a) dUMP can replace both moles of FdUMP that had been bound to the enzyme and, therefore, binds to both FdUMP sites; or (b) based on the observations cited above, there is only one catalytically functional site, with the remaining 1 mol of FdUMP bound to a nonfunctional site for which dUMP does not compete. If this is the case, then even high dUMP concentrations should be unable to prevent binding of 1 mol of FdUMP. The experiment depicted in Figure 1 shows that, if dUMP levels are raised sufficiently high (1 mM), the binding of FdUMP can be entirely prevented. These results are evidence against alternative b and indicate that dUMP does compete with FdUMP for both sites, although the apparent biphasic nature of the curve suggests that the relative affinities of the two sites for dUMP and FdUMP are not equal.

The Dissociation of Each Ligand (FdUMP or 5,10-CH_2H_4folate) from the Ternary Complex as a Function of the Concentration of the Other Ligand. The ligand binding method described provided the means to measure the slow reversal of a portion of the enzymatic reaction and thereby to obtain information about the order of dissociation of the reactants, FdUMP and 5,10-CH_2H_4folate. Experiments were carried out in which the effect of 5,10-CH_2H_4folate concentration on the dissociation of [^{3}H]FdUMP from the ternary complex was examined (Figure 2). It was found that the rate of exchange of [^{3}H]FdUMP from the [^{3}H]FdUMP-5,10-CH_2H_4folate-enzyme complex with unlabeled FdUMP was slowed 23-fold as the cofactor concentration was raised from 0 to 3 mM. The $t_{1/2}$ values from Figure 2 for dissociation of [^{3}H]FdUMP from the complex are a linear function of 5,10-CH_2H_4folate concentration over this range (plot not shown).

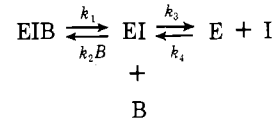
In the analogous experiment, the dissociation of [^{14}C]-5,10-CH_2H_4folate at various FdUMP levels was determined by measuring its exchange rate with 0.1 mM unlabeled cofactor in the medium (Figure 3). The rate of dissociation ($t_{1/2}$ = 38 min) was very similar to that of [^{3}H]FdUMP, and furthermore, remained constant regardless of FdUMP concentration, which was varied over the range of 0–160 μM.

These results are most easily explained by proposing an ordered mechanism of ligand binding for thymidylate synthetase in which FdUMP binds first to form a binary E-FdUMP complex which then reacts with 5,10-CH_2H_4folate to give the ternary complex. By the principle of microscopic reversibility, 5,10-CH_2H_4folate must dissociate first in the reverse reaction before FdUMP is free to leave its binding site on the enzyme. Thus, high concentrations of cofactor are able to suppress FdUMP dissociation, but FdUMP has no effect on cofactor dissociation. A reciprocal plot of the data in Figure 2 ($1/t_{1/2}$ vs. 1/(cofactor)) is hyperbolic (not shown) and extrapolates to zero at an infinite cofactor concentration (i.e., at

SCHEME I



SCHEME II



the ordinate where 1/(cofactor) = 0). Such a plot is diagnostic for the degree of order in the release of ligands from the ternary complex. A nonzero intercept would indicate some randomness to this dissociation, because it would show that some portion of the FdUMP can dissociate even at an infinite cofactor concentration.

The decomposition of the ternary enzyme-FdUMP-CH_2H_4folate complex in its simplest form is illustrated in Scheme I, where k_1 is the rate constant for release of 5,10-CH_2H_4folate (B) from the ternary EIB complex; k_3 is the constant for release of FdUMP (I) from the EI complex; and k_2 is the constant for combination of EI with B to give EIB. The expression for the net rate constant, k, for release of FdUMP from EIB is derived according to the partition analysis method of Cleland (1975):

$$k = k_1\left(\frac{k_3}{k_3 + k_2B}\right) + k_5 \qquad (4)$$

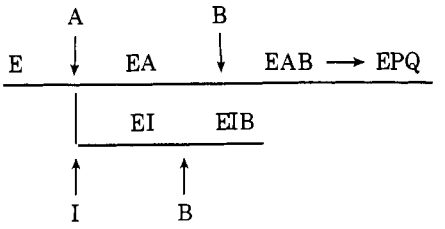
Since the data presented in Figure 2 indicates an ordered process in which FdUMP has a negligible rate of dissociation from the EIB complex, $k_5 = 0$. Equation 4 may then be rearranged to the reciprocal form.

$$1/k = 1/k_1(1 + k_2B/k_3) \qquad (5)$$

Equation 5 predicts a linear relationship between $1/k$ and B [5,10-CH_2H_4folate], which is, in fact, obtained if $t_{1/2}$ values from Figure 2 are plotted against cofactor concentration; an appreciable nonzero value for k_5 would result in a hyperbolic curve since there would then be a B term in the denominator. The vertical intercept of this linear plot (not shown) gives a value of 1.92×10^{-2} min^{-1} for k_1 (or $t_{1/2} = 36$ min) and reflects the slow back reaction. This value is equal, as it should be, to the experimentally determined value for FdUMP dissociation at zero 5,10-CH_2H_4folate.

The data presented in this paper, if combined with some previously published values, enables us to calculate the remaining rate constants for formation and dissociation of the ternary complex of enzyme, FdUMP, and 5,10-CH_2H_4folate. When considering the formation of the complex, Scheme I must be modified to one which contains a constant, k_4, for association of FdUMP with free enzyme (Scheme II). Santi et al. (1974b) measured the rate of formation of the enzyme-FdUMP-cofactor complex (EIB in Scheme II) as a function of FdUMP and obtained a rate constant of 2×10^7 M^{-1} min^{-1} at 24 °C, a value which they considered to be the apparent association constant of FdUMP with an enzyme cofactor (EB) complex. Myers et al. (1974) obtained the same number using a different technique, and we have obtained a value of 2.0×10^8 M^{-1} min^{-1} at 37 °C at a cofactor concentration of 0.1 mM. However, our data show that a kinetically important EB complex does not form, and the above value really represents a combination of several rate constants plus concentration terms for B:

SCHEME III



$$k_{on} = 2.0 \times 10^8 \text{ M}^{-1} \text{ min}^{-1} = k_4\left(\frac{k_2B}{k_3 + k_2B}\right) \qquad (6)$$

In this equation, the k_1 step of Scheme II is ignored because the conversion of EI to EIB would appear to be essentially irreversible when measuring the initial velocity of formation of the complex. The ratio k_3/k_2 is obtained from the horizontal intercept of a plot of $t_{1/2}$ values for dissociation of FdUMP against cofactor concentration (see eq 5) and is equal to 1.3×10^{-4}. By substituting $k_3 = 1.3 \times 10^{-4}k_2$ and B (cofactor) $= 1 \times 10^{-4}$ M into eq 6, we obtain a value for k_4, the association rate constant of FdUMP and free enzyme, of 4.6×10^8 M^{-1} min^{-1}. The ratio k_3/k_4 is really the K_{eq} for formation of the binary enzyme-FdUMP complex (EI in Scheme II). This number is obtained from the equilibrium dialysis data of Galivan et al. (1976) and has a value of 3.7×10^{-6} M. From this ratio, it is easily calculated that $k_3 = 1.7 \times 10^3$ min^{-1} and $k_2 = 1.3 \times 10^7$ M^{-1} min^{-1}.

Substrate Inhibition by 5,10-CH_2H_4folate Induced by BrdUMP. The interaction of FdUMP with thymidylate synthetase has been suggested to be a model for the enzyme reaction mechanism (Danenberg et al., 1974). If dUMP and FdUMP react in an analogous manner with the enzyme, the ordered mechanism indicated by the binding studies on the decomposition of the ternary covalent complex described in the previous section should also hold for the enzymatic reaction, i.e., steady-state kinetic studies should also be consistent with order. To test this prediction, we investigated the ability of an inhibitor competitive with dUMP to induce substrate inhibition by 5,10-CH_2H_4folate. Current theory of enzyme kinetics predicts (Fromm, 1967) that, in an ordered mechanism, a dead-end inhibitor competitive with the first substrate A (viz., combining only with free enzyme) would induce substrate inhibition by the second binding substrate B, provided the second substrate could combine with the EI complex. This situation is graphically represented by Scheme III, and the reaction velocity for induced substrate inhibition in an ordered mechanism is described by eq 7, using the nomenclature of Cleland (1963a-c).

$$v = \frac{VAB}{(K_{ia}K_b + K_aB)\,(1 + I/K_i(1 + B/K_{1b})) + K_bA + AB} \qquad (7)$$

From an inspection of Scheme III it is apparent that, as the concentration of substrate B is raised, the dissociation of inhibitor I will be hindered, since more of the enzyme will be diverted into the inactive EIB form. Due to the constraints imposed by the requirement for an ordered process, the inhibitor is not able to dissociate from the EIB complex (just as in the case of FdUMP in the presence of high concentration of cofactor). Furthermore, as the concentration of I is increased, the inhibition at any concentration of B will become stronger. However, in a random mechanism where neither substrate affects the dissociation of the other, substrate inhibition will not be observed, because the inhibitor is free to

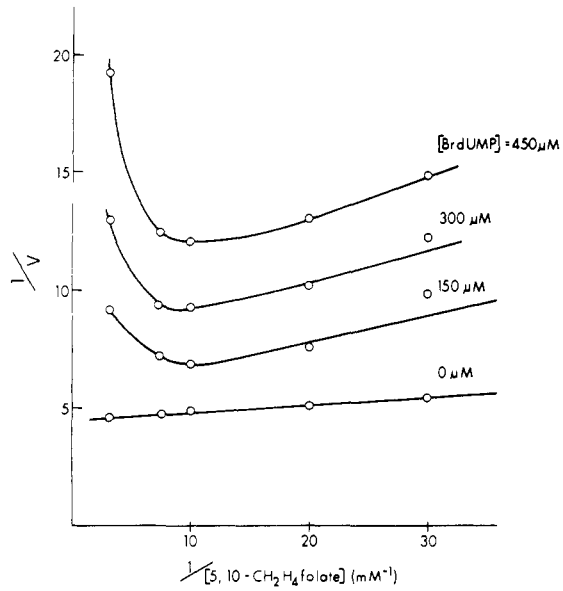


FIGURE 4: Substrate inhibition by 5,10-CH_2H_4folate in the presence of BrdUMP. 5,10-CH_2H_4folate was varied over the concentrations indicated at a dUMP concentration of 0.05 mM.

dissociate from EIB to give EB, which can react with A (dUMP) to give EAB. In more complex mechanisms involving preferred random or ordered pathways, partial substrate inhibition may be observed (Danenberg & Cleland, 1975).

BrdUMP was used as the dead-end inhibitor for these studies. Although BrdUMP had previously been shown to enter into an anomalous debromination reaction (Wataya & Santi, 1976) with thymidylate synthetase, this is a slow reaction (1–2% of the enzymatic reaction rate) and, therefore, should not be a significant factor when measuring initial reaction velocities. Nevertheless, we verified that the kinetics of inhibition by BrdUMP are competitive with respect to dUMP with a linear replot of slopes (figure not shown). A fit of these data to eq 2 for competitive inhibition gave a K_i value of 30 μM for BrdUMP and a K_a value of 1.2 μM for dUMP. When 5,10-CH_2H_4folate was varied at constant dUMP levels and variable BrdUMP levels, substrate inhibition by 5,10-CH_2H_4folate was observed (Figure 4), becoming especially pronounced at higher BrdUMP concentrations, where the curve appears to be asymptotic to the $1/v$ axis. The ordered model in Scheme III does, in fact, predict that the reaction rate should approach zero if the cofactor concentration is raised sufficiently. This was verified by plotting the cofactor concentration against $1/v$ in the region of substrate inhibition (0.3–0.9 mM 5,10-CH_2H_4folate) at fixed dUMP (20 μM) and BrdUMP (300 μM) levels. This plot was linear (not shown), which is consistent with a high degree of order in the mechanism; a significant contribution from a random pathway would result in partial substrate inhibition and a hyperbolic plot of $1/v$ vs. B, as observed previously for hexokinase (Danenberg & Cleland, 1975).

The set of constants which gave the best fit to eq 7 for the data in Figure 4 is as follows: K_a = 1.6 μM; K_{ia} = 16 μM; K_b = 5 μM; K_{ib} (the dissociation constant of B from EIB) = 75 μM; K_i = 30 μM.

According to the model in Scheme III the induced substrate inhibition by substrate B (cofactor) should also be competitive with the first substrate, since a greater concentration of A would increase EA and decrease the amount of enzyme in the EI form, thus decreasing the substrate inhibition. At the $1/v$ axis where the first substrate is at an infinite concentration, all the lines should meet to give a competitive pattern. Substrate inhibition resulting from another mechanism (e.g., binding to a secondary site) would give noncompetitive kinetics with respect to the first substrate. This experiment was carried out by varying dUMP from 0.1 to 2 mM at three inhibitory levels of cofactor (0.1, 0.2, and 0.3 mM) in the presence of a constant level of 300 μM BrdUMP. The result was a pattern which intersected at the $1/v$ axis, and a fit of the data to eq 2 for a competitive pattern gave a K_{is} value of 124 ± 47 μM (calculated k_{is} = 132 μM) and an apparent K_m of 21 ± 5 μM (calculated K_m = 28 μM).

Inhibition by 10-MeH_4folate. Cleland (1963c) has pointed out that, in an ordered mechanism, an inhibitor combining "downstream" from the variable substrate will give uncompetitive kinetics vs. that substrate, because, once the inhibitor combines with EA, there is no reversible connection between the enzyme forms E and EAI. This effect can also be viewed as a decrease in inhibition as the concentration of A is lowered, since the inhibitor combines only with EA. Thus, there is no inhibition of the slopes, resulting in parallel lines.

10-MeH_4folate was chosen as the cofactor analogue to be tested. Slavik & Zakrezewski (1967) showed that this compound is a good inhibitor of *E. coli* thymidylate synthetase, competitive with 5,10-CH_2H_4folate. We had previously found that 10-MeH_4folate displayed the same behavior with the *L. casei* enzyme ($K_i = 10^{-5}$ M) (Danenberg et al., 1974). When dUMP was varied from 0.67 to 500 μM in the presence of 0, 116, and 580 μM 10-MeH_4folate with cofactor fixed at 60 μM, an uncompetitive pattern resulted (not shown). To determine whether the inhibition was linear, the 10-MeH_4folate concentration was varied at fixed 5,10-CH_2H_4folate (60 μM) and very high dUMP levels (500 μM). The resulting plot of $1/v$ against inhibitor concentration was linear over a range of 10–200 μM 10-MeH_4folate (data not shown).

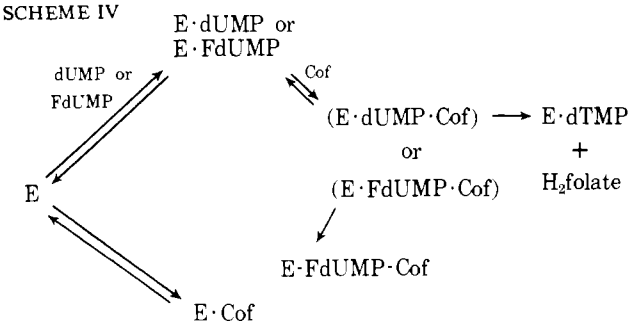
Substrate Inhibition by 5,10-CH_2H_4folate. Substrate inhibition by 5,10-CH_2H_4folate was also observed in the absence of BrdUMP. However, this inhibition by cofactor alone could only be seen at relatively high levels (>1 mM) and was not as pronounced as in the presence of BrdUMP. For example, at 1.2 mM 5,10-CH_2H_4folate, the reaction rate was inhibited 16% compared with the rate at 0.1 mM cofactor. In order to ensure that excess formaldehyde added with cofactor solution did not inhibit the enzyme, the same free formaldehyde levels were maintained in all reaction mixtures.

Discussion

Previous studies of substrate binding to thymidylate synthetase have resulted in several different mechanisms being proposed. Reyes & Heidelberger (1965), using enzyme isolated from Ehrlich ascites, and Blakley (1963), working with *S. faecalis* enzyme, have suggested ordered mechanisms in which the cofactor binds before dUMP. Lorenson et al. (1967) obtained data which did not allow them to differentiate random or ordered mechanisms, whereas Santi et al. (1976) have proposed a random mechanism for the thymidylate synthetase from *L. casei*. However, the evidence presented here is consistent with the mechanism of substrate binding for thymidylate synthetase from *L. casei* found in Scheme IV.

Scheme IV depicts an ordered mechanism in which enzyme–dUMP (or FdUMP) complexes are formed, which then add a molecule of cofactor and proceed either to form products in the presence of dUMP or to form the ternary covalent complex in the presence of FdUMP. No contribution from a random pathway was detectable within the sensitivity of the methods used in these experiments. The fact that measurements of FdUMP dissociation by a competitive ligand binding method led to the same conclusions about the mechanism as

SCHEME IV



kinetics studies of the forward reaction strengthens the hypothesis (Danenberg et al., 1974) that the catalytic events that lead to covalent binding of FdUMP are analogous to the initial steps of the enzymatic reaction.

Recent investigations in which binding of ligands was measured by equilibrium dialysis (Galivan et al., 1975, 1976) and changes in circular dichroism (Leary et al., 1975) also support Scheme IV for an ordered mechanism. These studies have shown dUMP, FdUMP, and dTMP bind to the enzyme with similar k_d values in the absence of folates; conversely, the binding of folate analogues is at least several orders of magnitude lower in the absence of nucleotides. No binding of 5,10-CH_2H_4folate could be detected under these conditions, but a binary complex between tetraglutamyl-H_4folate and the enzyme was sufficiently strong to be observed by the above methods, although the binding of the folate analogue was much weaker than in the presence of dUMP (Galivan & Maley, 1976). These studies, if taken at face value, suggest that some portion of the reaction flux, although small, may proceed through a random mechanism. However, the mere demonstration of binding does not prove that a complex is kinetically competent. The substrate inhibition that we observed with 5,10-CH_2H_4folate at high concentrations (>1 mM) shows that the cofactor at these levels forms an inhibitory complex with the enzyme, indicated in Scheme IV as a dead-end complex. There are several mechanisms by which this substrate inhibition could occur. The cofactor could bind at the active site first either to prevent binding of dUMP altogether, or to form an abortive E·cofactor·dUMP complex. Alternatively, the cofactor might be complexed to be a more weakly binding secondary site. This possibility is supported by previous findings of noncompetitive kinetics with a number of folate analogues including H_4amethopterin, H_4homofolate, and 5-MeH_4folate (Danenberg et al., 1974; Slavik & Zakrzewski, 1967). Further studies will be required to distinguish these alternatives.

One of our concerns in studying the kinetics of FdUMP exchange from the ternary complex and subsequent reaction of the enzyme has been the question of the nonidentity of two binding sites. The first-order dissociation curves that we obtained were linear to 10% of the original binding, which suggests a single class of binding sites for the release of ligands. We were able to show that dUMP competes with FdUMP for both sites (Figure 1) but that very high dUMP concentrations are required to exclude FdUMP binding entirely. Computer simulation of the biphasic curve in Figure 1 indicates about a tenfold difference in the relative affinities of the two FdUMP binding sites for dUMP. Thus, at least for the formation of the initial enzyme–nucleotide binary complexes, the sites appear to be nonequivalent, in general agreement with the data of Galivan et al. (1976). A hypothesis which might explain these apparent discrepancies is that the two sites are identical in their catalytic parameters once filled with substrates but function in an alternating, "flip-flop" manner (Lazdunski et al., 1971), so that initially the nucleotide encounters an asymmetric arrangement of subunits.

The suppression of FdUMP dissociation by 5,10-CH_2H_4folate is a consequence of the ordered mechanism of the enzyme and may be of significance to cancer chemotherapy with 5-FU. The regeneration rate of active thymidylate synthetase from the inactive complex in 5-FU-treated tumor cells has so far not been considered as one of the factors which might have a part in determining the overall chemotherapeutic effectiveness of 5-FU. In fact, once the enzyme encountered FdUMP it had been generally considered as permanently inactivated, the tissue recovering from the effects of the drug by synthesis of new enzyme molecules (Myers et al., 1975). Our data show that at the low cofactor concentrations that have been found in tumor tissues (ca. 3 μM in L1210 cells (Moran et al., 1976)), the recovery of enzyme activity can be reasonably rapid in the presence of sufficient dUMP, and probably comparable with the rate of biosynthesis of new enzyme molecules. Since increasing the cofactor concentration effectively decreases the dissociation constant of FdUMP and thus makes it a more effective inhibitor, there is a clear implication that sustaining high H_4folate levels in cells may increase the chemotherapeutic efficacy of 5-FU by lengthening the time that enzyme activity is inhibited. Indeed, experiments have been recently reported (Ullmann et al., 1978) in which addition of folinic acid (an exogenous source of reduced folate) to folate-depleted cells in culture increased the cytotoxicity of 5-FU. However, this effect remains to be demonstrated in vivo.

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Mechanism of Pigeon Liver Malic Enzyme. Reactivity of Class II Sulfhydryl Groups as a Conformational Probe for the "Half-of-the-Sites" Reactivity of the Enzyme with Bromopyruvate†

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ABSTRACT: A method is described for the selective masking of nonessential SH groups of pigeon liver malic enzyme by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) in the presence of $NADP^+$, Mn^{2+}, and the substrate analogue tartronate. The resulting enzyme derivative containing four intact class II SH groups per tetramer is fully active in the oxidative decarboxylation of malate. Alkylation of two class II SH groups by the affinity label bromopyruvate inactivates this enzyme and abolishes the reactivity of the two remaining groups toward this reagent, confirming the "half-of-the-sites" behavior reported in a previous communication (Chang, G. G., & Hsu, R. Y. (1977) *Biochemistry 16,* 311–320). In contrast, "all-of-the-sites" reactivity is obtained for DTNB, NEM, iodoacetate, and iodoacetamide, which cause inactivation by reacting with all of the class II SH groups. The reaction of the enzyme derivative with DTNB or NEM follows a pseudo-first-order process, yielding second-order rate constants of 0.49 and 0.13 mM^{-1} min^{-1}, respectively. The rate constant of DTNB is unaffected by partial modification of the enzyme with other "all-of-the-sites" reagents, whereas the rate constants of both reagents with the enzyme which has been exhaustively alkylated by bromopyruvate are decreased by 2.4-fold for DTNB and 3.6-fold for NEM. The reaction of partially alkylated malic enzyme containing fewer than two bound pyruvyl residues per tetramer exhibited biphasic behavior, which can be accounted for by two parallel pseudo-first-order processes with rate constants corresponding to those of the unalkylated and dialkylated enzyme. The "half-of-the-sites" effect of bromopyruvate is interpreted on the basis of negative cooperativity resulting from specific conformation changes induced by the alkylating ligand.

Pigeon liver malic enzyme (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) is a tetramer composed of identical or nearly identical subunits (Nevaldine et al., 1974). In previous studies we have shown that this enzyme contains four independent and equivalent nucleotide binding sites (Hsu & Lardy, 1967a). The substrates $NADP^+$ and malate exhibit typical Michaelis–Menten (i.e., noncooperative) kinetic behavior in the oxidative decarboxylase reaction at a constant level of metal activator (Hsu et al., 1967). The initial velocity and product inhibition patterns are consistent with a sequential, ordered kinetic mechanism with $NADP^+$ adding first, followed by malate, and the release of CO_2, pyruvate, and NADPH as products (Hsu et al., 1967). More recently, the possibility of anticooperativity or nonidentical active sites is suggested by the following observations: (a) metal binding studies indicating the presence of two tight and two to four weak Mn^{2+} sites per enzyme tetramer (Hsu et al., 1976); (b) the apparent kinetic negative cooperativity of Mn^{2+}, potentiated by the substrate malate (Hsu et al., 1976); (c) a transient burst of enzyme-bound NADPH which equals approximately half of the active-site concentration (Reynolds et al., 1978); and (d) the "half-of-the-sites" reactivity of bromopyruvate, which inactivates the enzyme after alkylating two of the four "essential" SH groups (Chang & Hsu, 1977).

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