Spectroscopic Studies of Ternary Complexes of Thymidylate Synthetase, Deoxyribonucleotides, and Folate Analogs*  

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Conformational changes accompanying the formation of binary and tightly bound ternary complexes of thymidylate synthetase and all possible combinations of three folate analogs (N-10-ethyl-quinazoline, folic acid, 2'-deoxyribonucleotide, and folic acid) and three deoxyribonucleotides (5-fluoro-2'-deoxyuridylic acid (FdUMP), 2'-deoxyuridylic acid (dUMP), and thymidylic acid (dTMP)) were studied by means of ultraviolet difference spectroscopy. The amplitudes of the spectral changes upon ternary complex formation were 2-3-fold greater than those generated by formation of binary enzyme-nucleotide and enzyme-folate analog complexes. Difference spectra of the ternary complexes all showed a major increase in absorbance in the region of 320-340 nm, presumably due to perturbations of the folate analog chromophores, whereas decreases in absorbance occurred over a range of 260-310 nm. N-10-ethyl-quinazoline tended to form the complex with the greatest filtration efficiency on nitrocellulose filters, followed by folic acid triggulamate and folic acid, whereas among the nucleotides, the most stable complexes were formed with FdUMP, followed by dUMP and dTMP. A correlation was observed between the apparent stability of the ternary complex and the magnitude of the absorbance change in its difference spectrum. The formation of the various ternary complexes showed three different categories of rate behavior: 1) very rapid formation of the complex; 2) biphasic formation with a rapid phase and a slow phase requiring up to 90 min for completion; and 3) in the case of the ternary complex formed with enzyme, FdUMP, and folic acid, only a slow phase of binding. The slow formation of the latter complex was accompanied by concomitantly slow changes in the difference spectrum. However, in those cases of biphasic formation of the complexes, almost all of the spectral change occurred rapidly, and very little of it corresponded to the slow phase of complex formation. To accommodate these observations, a model is proposed involving a sequential interaction of the two subunits of thymidylate synthetase.

Thymidylate synthetase (EC 2.1.1.45), which catalyzes the formation of dTMP1 and H4PteGlu from dUMP and 5,10-CH3H4PteGlu, has been the object of numerous studies because of its importance in cancer chemotherapy as a target enzyme for metabolic inhibitors and because of certain unique features of the enzymatic mechanism. Of particular significance in both regards is the interaction of FdUMP, a metabolite of the cancer chemotherapeutic drug FURa, and 5,10-CH3H4PteGlu with the enzyme to form a tightly bound (Kd 10^{-11} M) covalent ternary complex (1-3). The unusual aspect of this complex is that the tight binding of the ligands to the enzyme is not derived simply from an adsorption to the binding sites. Subsequent to binding, a chemical reaction occurs that results in the formation of a covalent sulfide bond connecting the enzyme to the C-5 position of FdUMP and a methylene bridge connecting the H4PteGlu to the C-5 position of the nucleotide (1). The considerable interest in this complex has stemmed from the realization that the chemical modification of these compounds by the enzyme occurs because they are substrates for a portion of the normal catalytic sequence; the complex, therefore, can be considered to be a frozen facsimile of an intermediate of the methylene transfer reaction carried out by thymidylate synthetase. In this paper, we will use the term "catalytic complex" to distinguish from enzyme-inhibitor adsorption complexes the type of inhibitory complexes that are formed by the catalytic action of the enzyme.

Further studies since the discovery of the thymidylate synthetase-FdUMP-5,10-CH3H4PteGlu complex have shown that, although its covalent structure is of great significance for elucidation of the chemical mechanism of the enzyme reaction, the covalent binding per se is not the major factor determining the stability or the "tightness" of this type of complex, as is the case with enzyme inhibitors of the alkylating affinity label type. Studies of secondary isotope effects indicate that the rate-determining step in the release of FdUMP from the complex is not the enzyme-catalyzed cleavage of the covalent bond joining the nucleotide to the enzyme, but comes after this chemical reaction. Folate analogs not chemically capable of forming the covalent methylene bridge to the nucleotide (e.g. 10-methyl H4PteGlu) also induce the tight binding to the enzyme not only of FdUMP (1) but of other deoxyribonucleotides as well such as the normal substrate dUMP (4), presumably by participating in the formation of tightly bound ternary complexes that include a non-

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covalently bound folate molecule. The complexes formed with the folate analogs, however, are not of equal stability. Some dissociate very rapidly, whereas others are quite stable; for example, FdUMP that has been bound to the enzyme in the presence of PteGlu dissociates more slowly than from the covalent complex formed with 5,10-CH₂H₄PteGlu (6). Fluorine-19 nuclear magnetic resonance studies have directly shown that folate analogs cause a substantial shift of enzyme-bound FdUMP toward the form of the covalent 5,6-dihydropyrimidine adduct with the sulfhydryl group of the enzyme (6), thereby demonstrating that a catalytic processing of the ligands occurs in these complexes just as it does in the enzyme-FdUMP-5,10-CH₂H₄PteGlu complex.

These results strongly suggest that conformational changes induced in the enzyme by the folate analog may be an integral part of the formation of tightly bound ternary complexes. In fact, a number of studies have shown that substantial shifts occur in the ultraviolet spectrum upon binding of 5,10-CH₂H₄PteGlu to the binary thymidylyltransferase-FdUMP complex to form the ternary complex (3). We confirmed by means of hydrodynamic analysis (7) that these spectral changes are indeed indicative of a major conformational change in the enzyme, the extent of which is dramatically illustrated by the fact that the free enzyme is readily separable from the ternary complex on a Sephadex G-100 column (100 cm).

Because ternary complex formation very likely is analogous to a normal catalytic event, it is reasonable to assume that the conformation of the enzyme when in the ternary complex form is also closely analogous to that of a normally transient catalytic conformation (7). The magnitude of the conformational change accompanying ternary complex formation could then be regarded as striking evidence that the catalytic process is accompanied by conformational changes distinct from, and much larger than, those accompanying the adsorption of substrates to their binding sites. The idea that changes in conformation have an essential role in the catalytic function has been incorporated into various models of enzyme action (8-10), but has not yet been proven because of the difficulty of designing experimental systems that allow the direct monitoring of conformational changes in the enzyme as the enzyme reaction is proceeding. However, if, as the evidence suggests, the tightly bound thymidylyltransferase-nucleotide-folate analog complexes are formed as a result of catalytic processes, they may provide a novel system for studying the relationship between conformational change and catalytic function. It was, therefore, of interest to us to investigate the nature of these complexes further. In the present study, we used ultraviolet difference spectroscopy to determine the degree of conformational change in the enzyme accompanying the formation of ternary complexes of thymidylyltransferase and various folate analogs and nucleotides, and to determine how closely the change in enzyme conformation is associated with the stability and rate of formation of the ternary complex. We found that 1) the magnitudes of the spectral changes when complexes are formed with folate analogs are comparable to those obtained using 5,10-CH₂H₄PteGlu; 2) a correlation exists between the stability of the complex and the magnitude of conformational change; and 3) a slow change in absorbance in one case closely corresponded to the rate of formation of an isolable complex, while in several cases it did not. A model is proposed to account for the data.

**EXPERIMENTAL PROCEDURES**

*Materials*—[6-3H]FdUMP (18 Ci/mmol), [2-14C]FdUMP (52 mCi/mmol), [6-3H]dUMP (13 Ci/mmol), [2-14C]dUMP (56 mCi/mmol), [2-14C]dTMP (55 mCi/mmol), and [methylene-3H]dTMP (45 Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA. [3',5'-7,9-3H]Folic acid, potassium salt (29 Ci/mmol), was obtained from American Corp. and repurified by paper chromatography, using pyridine/n-butyl alcohol/water (1:1:1) as the solvent. NEQ was a generous gift of Dr. T. R. Jones of the Institute of Cell and Research, Sutton, Surrey. PteGlu was provided by Dr. Richard G. Moran, Children's Hospital of Los Angeles, Los Angeles, CA. 1-1-1-(+)5,10-CH₂H₄PteGlu and FdUMP were synthesized as described previously (10). Other biochemicals were purchased from Sigma.

*Thymidylyl Transferase*—Homogeneous enzyme was obtained from methotrexate-resistant Lactobacillus casei (11) and was dialyzed every 3 days in 50 mM MOPS buffer, pH 7.4, containing 250 mm KCl and 2 mm dithiothreitol. Enzyme concentration was determined spectrophotometrically by measuring absorbance at 280 nm using an extinction coefficient of 108,000 (12), and also as 1/1.7 pmol of [3H]FdUMP bound in the presence of 5,10-CH₂H₄PteGlu and FdUMP (7). The dialyzed enzyme was routinely assayed spectrophotometrically (13) to determine that there was no loss of enzyme activity.

*Difference Spectra*—For most experiments, samples were prepared by adding 410 μl of enzyme to one compartment of each of two semimicro split cuvettes (1 cm) and an equal volume of solution containing the other ligands to each other compartment. Final enzyme concentration was about 3.5 μM, and the concentration of the ligands was three times greater, or about 11 μM. For difference spectra of the enzyme-nucleotide binary complexes, the enzyme and nucleotide concentrations were increased to 5.6 and 19 μM, respectively. In every case, the concentration of MOPS, KCl, and dithiothreitol in all cuvette compartments was made identical to that of the dialyzed enzyme. Spectra were scanned in the double-beam mode on a Model 25 Beckman Recording Spectrophotometer at ambient temperature (~25°C) between 440 or 400 and 240 nm, using both the tungsten and deuterium light sources, to determine the base-line. The contents of the two compartments in the sample cuvette were thoroughly mixed by disposable pipettes, and the difference spectra were recorded periodically for at least 90 min. Then, the contents of the reference cuvette were likewise mixed, and the spectra were recorded periodically as before. Spectra were usually recorded at a rate of 20 mm/min. The standard deviation of the combined enzyme and ligands versus water was no greater than 2.0 A.

*Binding Studies*—These experiments were conducted with the same concentrations of dialyzed enzyme, ligands, and other compounds of the reaction mixture as used for the spectral determinations. After preincubation of the enzyme and ligands separately at 25°C, an aliquot of the ligand solution containing radiolabeled nucleotide was added to the enzyme in a total volume of 350 μl to begin the experiment. Aliquots (18 or 22 μl) were filtered periodically through nitrocellulose discs (14) and washed twice with ice-cold 50 mM Tris-HCl, pH 7.4, for determination of bound radioactivity.

**RESULTS**

*Difference Spectra*—The spectra shown here are representative ones of at least two determinations for each of the ligand combinations. The amplitudes of the peaks did not vary by more than 10% of the absorbance change between duplicate spectra obtained using the same solutions. Mixing the compartments of the reference cuvette after determining each spectrum resulted in the disappearance of the peaks.

Spectra of complexes between thymidylyl transferase, FdUMP, dUMP, and dTMP are shown in Fig. 1. The spectra differed considerably in the region of 280–300 nm, but all showed decreases in absorbance in the region below 270 nm. The difference spectra of thymidylyl transferase with NEQ and with nucleotides plus NEQ are shown in Fig. 2. The enzyme-NEQ binary complex difference spectrum exhibited a maximum at 332 nm, a red shift of about 20 nm of the long wavelength maximum of NEQ, and a minimum at 284 nm. The difference spectra of the ternary complexes with FdUMP, dUMP, and dTMP were all very similar, with maxima at 334–336 nm and 274–278 nm and minima at 303–305 and 254–257 nm. The differential extinction coefficient for the largest maximum was more than twice as large as that for the binary complex maximum. No changes in the magnitude...
of the absorbance were noted for any of these difference spectra over a period of 90 min.

Similar experiments were performed with PteGlu₂ (Fig. 3). The enzyme-PteGlu₂ binary complex difference spectrum had a maximum at 317 nm and a minimum at 280 nm. All the difference spectra of the enzyme-nucleotide-PteGlu₂ ternary complexes had maximum absorbance in nearly the same region (319–320 nm) as the binary complex, but with several times the magnitude. Minima were observed for the ternary complexes with FdUMP, dUMP, and dTMP in the region of 258–265 nm, which is somewhat blue-shifted from the absorption maxima of each of the nucleotides. The region between the maximum and minimum of each of these ternary complexes was considerably more differentiated than that observed for the analogous complexes with NEQ.

In order to examine the effects on the difference spectra of the same folate less two glutamyl groups, comparable spectra were obtained with PteGlu (Fig. 4), which is known to partic-

**Fig. 1.** Difference spectra of the interaction of nucleotides with thymidylate synthetase. Enzyme plus FdUMP (---), dUMP (--), or dTMP (--).

**Fig. 2.** Difference spectra of the interaction of NEQ with thymidylate synthetase. A, spectrum of NEQ; B, difference spectrum of the enzyme-NEQ complex; and C, difference spectra of enzyme, NEQ, and FdUMP (---), dUMP (--), or dTMP (--).

**Fig. 3.** Difference spectra of the interaction of PteGlu₂ with thymidylate synthetase. A, spectrum of PteGlu₂; B, difference spectrum of the enzyme-PteGlu₂ complex; C, difference spectra of enzyme, PteGlu₂, and FdUMP (---), dUMP (--), or dTMP (--).

**Fig. 4.** Difference spectra of the interaction of PteGlu with thymidylate synthetase. A, difference spectrum of the enzyme-PteGlu complex; B, difference spectra enzyme, PteGlu, and FdUMP (---), dUMP (--), or dTMP (--).
ipate in less tight binding than PteGlu, with thymidylate synthetase either in the presence or absence of nucleotides (5). The enzyme-PteGlu and the enzyme-PteGlu<sub>3</sub> binary difference spectra were similar in shape although the largest absorption differences of the former are about one-half those of the latter.

Although the enzyme-FdUMP-PteGlu and the enzyme-FdUMP-PteGlu<sub>3</sub> difference spectra were similar, the spectra of the complexes formed with PteGlu and each of the nucleotides were quite different, except in the region of 320 nm, where a maximum is observed in all three different spectra. A unique feature of the thymidylate synthetase-FdUMP-PteGlu difference spectrum was the time-dependent increase in the absorbancies, as measured by both the increase at 317 nm and the decrease at 267 nm. The time course of this increase, as measured by the difference in the absorbance between the maximum and minimum, is shown in Fig. 5.

**Nitrocellulose Disc Filtration of Complexes**—In order to quantitate the amount of radiolabeled ligand bound by the nitrocellulose filtration method (13), the filtration efficiency, i.e. the percentage of bound radiolabeled ligand which is detectable after filtration and washing, must be determined (15). This procedure requires that there be a high ratio of protein to ligand such that virtually all of the ligand is complexed, and this is demonstrated experimentally when further additions of protein do not increase the amount of ligand retained by the filter (15). Using filtration efficiencies for complexes with <sup>3</sup>H-nucleotides and NEQ, PteGlu, and PteGlu<sub>3</sub> that had been reported previously (5), the molar ratios of bound nucleotide/enzyme under conditions in which the difference spectra were taken could be determined (Table I).

We measured the time-dependent binding of thymidylate synthetase to the various ligand combinations by the nitrocellulose filtration method, and surprisingly, found that some of the ternary complexes required many minutes or even hours for maximal binding of radiolabeled nucleotide. Each of these binding experiments was repeated at least once. As can be seen in Fig. 6A, the enzyme-dUMP-NEQ complex required about 1 h to achieve apparent maximal binding, although no concomitant changes were observed in the difference spectra for this time period. The analogous FdUMP and dTMP complexes showed no apparent time-dependent increases over a 2–3 h period after the initial binding, but filtration of the complexes after 24 h showed a slight increase of binding (Table I).

In the case of nitrocellulose-filtrable binding of nucleotides

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**FIG. 5.** Increase of the differential absorbance of the enzyme-FdUMP-PteGlu complex with time. A<sub>ΔΔε</sub> represents the difference between the extinction coefficients of the positive (317 nm) and negative (288 nm) peaks.

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**TABLE I**

<table>
<thead>
<tr>
<th>Folate analog</th>
<th>FdUMP</th>
<th>dUMP</th>
<th>dTMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEQ (2 h)</td>
<td>1.2</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>(24 h)</td>
<td>1.6</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>(69%)</td>
<td>(66%)</td>
<td>(66%)</td>
<td></td>
</tr>
<tr>
<td>PteGlu&lt;sub&gt;3&lt;/sub&gt; (2 h)</td>
<td>1.3</td>
<td>0.79</td>
<td>0.99</td>
</tr>
<tr>
<td>(66%)</td>
<td>(46%)</td>
<td>(22%)</td>
<td></td>
</tr>
<tr>
<td>PteGlu (2 h)</td>
<td>0.87</td>
<td>0.69</td>
<td>0.62</td>
</tr>
<tr>
<td>(63%)</td>
<td>(41%)</td>
<td>(30%)</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 6. Rates of formation of ternary complexes of thymidylate synthetase, nucleotides, and folate analogs.** Binding to thymidylate synthetase of [<sup>14</sup>C]FdUMP (A), [<sup>14</sup>C]dUMP (Δ), or [<sup>14</sup>C]dTMP (C) in the presence of A, NEQ, B, PteGlu, or C, PteGlu, as a function of time was determined by nitrocellulose disk filtration. The enzyme concentrations were 3.6 μM for A and B, and 3.5 μM for C. Aliquots (18 μl) were filtered.

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Stoichiometry of binding to thymidylate synthetase in the presence of NEQ, PteGlu<sub>3</sub>, or PteGlu and filtration efficiency of the complexes

Enzyme (3.5 μM) was incubated with 11 μM ligands for the times indicated, and aliquots were filtered periodically as described under "Experimental Procedures." For PteGlu and PteGlu<sub>3</sub>, there was no significant increase in binding after 24 h. Each figure represents the average of at least two experiments. The figures in parentheses represent the observed filtration efficiency for each complex and were taken from Ref. 5.

As can be seen from a comparison of Fig. 5 and the time course of FdUMP binding in Fig. 6C, the rate of change in the absorbance closely parallel the rate of binding of FdUMP.

**DISCUSSION**

Spectra of Binary Enzyme-Nucleotide and Enzyme-Folate Complexes—The ultraviolet difference spectrum of the binary thymidylate synthetase-FdUMP complex has been previously studied by Lewis et al. (16). These workers observed a broad trough in absorbance centered at 275 nm, and because this absorbance change is closer to the maximum of tryptophan (275 nm) than to that of FdUMP (269 nm), it was attributed to tryptophan perturbations, rather than to loss of the fluo-
rouracil chromophore resulting from addition of the sulfhydryl group. Such a decrease is anticipated on the basis of fluorine-19 nuclear magnetic resonance studies which show that about 25% of the enzyme-bound FdUMP is in the form of a covalent adduct with the catalytic sulfhydryl group of the enzyme (6). The difference spectrum that we obtained for this complex (Fig. 1) is similar to that obtained by Lewis et al. (16), except that the trough in our spectrum appears to be centered at about 268 nm, and therefore, we cannot exclude the possibility that at least a portion of this decrease is due to saturation of the 5,6-double bond of FdUMP. However, the total absorbance change at this wavelength is about 4-fold greater than would be expected, based on the assumption of one saturated site with 25% of the nucleotide covalently bound. This result indicates either that under our conditions all of the nucleotide in the binding site is in the covalent adduct form or that other factors contribute to the absorbance changes in this region. The difference spectra of thymidylate synthetase-dUMP and -dTMP complexes also show decreases in this region, centered, respectively, at about 258 nm, which is close to their absorbance maxima, indicating that dUMP and dTMP may also undergo a covalent interaction with the enzyme. There are, in addition, small increases in absorbance for the dUMP and FdUMP complexes at 280–290 nm, which can be attributed to tryptophan perturbations (17).

Exposure of the enzyme to the folate analogs in the absence of nucleotides caused a difference spectrum to be generated, indicating the formation of binary enzyme-folate complexes (Figs. 2B, 3B, and 4B). This finding is interesting because kinetics studies have shown an ordered mechanism for thymidylate synthetase in which the nucleotide is the obligatory first-binding substrate both in the enzyme reaction and in the formation of the ternary complex with FdUMP and 5,10-CH\(_2\)\(_2\)H\(_2\)PteGlu (18). Thus, one might not a priori expect to observe binding of folates to the free enzyme. The nature and function of such complexes remains to be elucidated (their overall shapes depend to a considerable extent on the probable tendencies of these nucleotides to form the 5,6-dihydro derivative with the active site sulfhydryl group). The spectra of these tightly bound ternary complexes regardless of whether or not a covalent bond is formed from the folate component to the nucleotide.

An obvious characteristic of the difference spectra is that their overall shapes depend to a considerable extent on the folate analog used to form the complex. Thus, the spectra generated by NEQ are substantially different from those of PteGlu\(_3\), not only in the high wavelength region above 300 nm, where the absorbance change is most likely owing to perturbation of the folate chromophore, but also in the region below 300 nm which is most likely to reflect changes in the environments of aromatic amino acid side chains (17). These observations indicate that the conformational change in the enzyme upon catalytic complex formation is not entirely predetermined, and at least a substantial portion of it occurs in response to certain structural features of the folate component. It is not clear whether these different conformational states represent varying degrees of transition along the normal catalytic pathway or could also be abnormal structures that are not formed during the course of the enzyme reaction.

It is interesting that the spectra of complexes involving NEQ do not have the substantial decreases in absorbance below 300 nm that are characteristic of those obtained with PteGlu and PteGlus. This observation is not consistent with the anticipated loss in absorbance at 260–270 nm that would result from addition of the catalytically active sulfhydryl group to the pyrimidine groups of the nucleotides. However, we hesitate to propose that this well-established reaction is not occurring in the presence of NEQ. It may be that a decrease in absorbance in this region is being obscured by absorbance changes in the opposite direction arising from amino acid residues.

This study required a group of folates and nucleotides which in various combinations would form catalytic complexes of different stabilities so that the degree of correlation between this catalytic parameter and conformational change could be determined. In this study, we used the filtration efficiency of the complexes or nitrocellulose filters as a facile way of assaying their relative stabilities. The filtration efficiency of an enzyme-ligand complex has been shown to be a function of its dissociation constant (15). Relative filtration efficiencies (Table I) indicate that NEQ forms the most stable complexes, followed in order by PteGlus and PteGlu. The relationship between the stability of a complex and its filtration efficiency is further supported by the observation that the apparent binding stoichiometries (Table I) as well as the rate constants for dissociation (k\(_{\text{off}}\) values) of the complexes (5) are in the same order as are their respective filtration efficiencies. The tight complexes formed with NEQ as the folate component all have about the same stability regardless of which nucleotide is used. However, the stabilities of the complexes formed in the presence of PteGlu and PteGlus appear to be determined to some extent by the structure of the nucleotide component, with FdUMP giving rise to the most stable complex, followed by dUMP and dTMP. It is interesting to note that the stabilities of the latter complexes are in the same order as are the probable tendencies of these nucleotides to form the 5,6-dihydro derivative with the active site sulfhydryl group.

These relative stabilities of the catalytic complexes are reflected in the magnitudes of the respective difference spectra. This is especially apparent when comparing the difference spectra generated in the presence of PteGlu and PteGlus, both of which have the same chromophore. The enzyme-FdUMP-
PteGlu and -PteGlu₃ complexes, which the filtration efficiency measurements indicate to be of about the same stability, also have similar shapes and similar changes in absorbance. However, when dUMP and dTMP are the nucleotide components, complexes formed in the presence of PteGlu are weaker than those formed with PteGlu₃ and also generate spectra of a considerably lower amplitude than do the corresponding complexes formed with PteGlu₃. The spectra obtained with all three nucleotides in the presence of NEQ are very similar, consistent with the almost identical stabilities of these complexes. These observations indicate a close association between the tightness of the ternary complexes and the extent of conformational change in the enzyme. Thus, if the formation of a more stable complex in the presence of a particular folate analog is a manifestation of greater catalytic activity, these data also provide evidence for an involvement of conformation change in the catalytic function of the enzyme. This conclusion is consistent with the suggestion of Yaneelev and Koshland (23) that a better substrate will cause a larger change in conformation.

**Correlation between Complex Formation and Spectral Change**—From Fig. 6 it is apparent that the binding curves for the nucleotides fall into three distinct categories which indicate 1) rapid formation of tightly bound complexes where the maximum is reached before the first point can be assayed and no further increase over the indicated time periods is observed; 2) slow, monophasic complex formation which starts from zero and is approximately linear with time; and 3) biphasic formation of ternary complexes with a rapid phase corresponding to about half of the maximal binding and a slow phase requiring many minutes for completion. Since thymidylate synthetase consists of two subunits with a binding site on each one, the biphasic binding phenomenon suggests the rapid formation of the catalytic complex in one of the two sites and slow formation of the complex in the other. This hypothesis is consistent with much data indicating that thymidylate synthetase from L. casei has binding sites which are not identical with respect to ligand binding (1–3). For example, a Scatchard plot of the binding of dUMP and PteGlu₃ shows a difference in affinity of 100-fold between the two sites (4). The aspects of these data that have to be reconciled are that 1) not all combinations of ligands produce the biphasic binding curves, which they should do if it were merely a matter of binding to two nonequivalent sites; and 2) there is little or no time-dependent spectral change concomitant with the slow-binding phase of the biphasic complexes, which does not appear to be consistent with a close association between the tight binding of the ligands and conformational change. A satisfactory accommodation of the binding curves and the spectral changes may be provided if we invoke three previously proposed concepts: 1) when the folate binds to the enzyme-nucleotide-binary complex, a loosely bound ternary adsorption complex is formed which becomes converted to the tightly bound catalytic complex by means of rate-determining conformational changes (2); 2) the structures of the ligands influence the conformational response of the enzyme; and 3) the subunits interact in a sequential manner such that the formation of the ternary complex in the first accessible site induces conformational changes that permit binding in the second, initially inaccessible site (24). With regard to the latter, it has been observed in studies using both spectral changes and hydrodynamic methods (7) as conformational probes that about 80% of the total conformational change occurs upon tight binding of one enzyme equivalent of FdUMP and 5,10-CH₂H₄folate (corresponding to the filling of one site). This observation is consistent with the present data showing little or no further conformational change accompanying the slow-binding phases of the biphasic curves and suggests that the binding of ligands in the first site serves not only to open the second site, but also to cause that subunit to assume a conformation close to the catalytic one. These concepts are summarized in Equations 1–4.

\[ \text{Nucl} + E \rightleftharpoons \text{Nucl} \cdot E \]  
\[ \text{Nucl} \cdot E + \text{fol} \rightleftharpoons \text{Nucl} \cdot \text{fol} \cdot E \overset{\kappa_1}{\rightarrow} \text{Nucl} \cdot \text{fol} \cdot E = E^* \]  
\[ \text{Nucl} \cdot E^* \rightleftharpoons \text{Nucl} \cdot E^* \]  
\[ \text{Nucl} \cdot E^* + \text{fol} \rightleftharpoons \text{Nucl} \cdot \text{fol} \cdot E^* \overset{\kappa_2}{\rightarrow} \text{Nucl} \cdot \text{fol} \cdot E^* \]

In this scheme, E represents the free enzyme, while \( E^* \) represents the 1:1:1 nucleotide-folate-thymidylate synthetase complex. The adsorption complexes are indicated by the dotted lines, and the tight-binding complexes by the solid lines. \( K_1 \) and \( K_2 \) are the dissociation constants of the respective enzyme-nucleotide complexes, and \( k_1 \) and \( k_2 \) are the catalytic constants leading to formation of the tightly bound ternary complexes. The rapid, monophasic binding is easily accounted for by assuming that the tightly bound complexes form at the same rate in both sites or at a faster rate in the second site (that is, \( K_1 = K_2 \) and \( k_1 = k_2 \), or \( k_2 > k_1 \)).

The biphasic binding curves, which indicate a low \( V_{\text{max}} \) for formation of the ternary complex in the second site, could result 1) if the optimal conformational change required to completely open the second site did not occur with certain combinations of folate analogs and nucleotides, resulting in hindered binding to the second site \( (K_2 \gg K_1) \), and thereby a low concentration of the adsorption complex, nucleotide-\( E^* \); or 2) if the nucleotide and folate bind normally to the second site \( (K_1 = K_2) \), but the conformational change that has occurred from binding in the first site is abnormal in such a way as to give a low catalytic capability for formation of the tightly bound complex in the second site \( (k_2 < k_1) \).

The binding profile of FdUMP in the presence of PteGlu (Fig. 6C) is unique in that the nucleotide binds slowly to the enzyme without any discernible rapid-binding phase, suggesting that in this case, formation of the ternary complex in the first site is slow (instead of only in the second site, as with the other two folates). This situation could result either from weak binding of the ligands in the first site (large \( K_1 \)) or from a low value for the catalytic constant \( k_1 \). The latter possibility is reasonable because the low stability of complexes formed with PteGlu (the \( t_{1/2} \) for dissociation is less than 1 min) (5) shows that the ability of this folate to induce the proper conformational changes leading to tight binding is poor. If this idea is correct, formation of this particular complex in parallel with time-dependent changes in its difference spectrum (cf. Figs. 5 and 6C) constitutes evidence for a direct link between the major conformational changes in the enzyme and formation of the catalytic complex in the first site.

**REFERENCES**


**Spectra of dTMP Synthetase-Nucleotide-Folate Complexes**
Spectra of dTMP Synthetase-Nucleotide-Folate Complexes