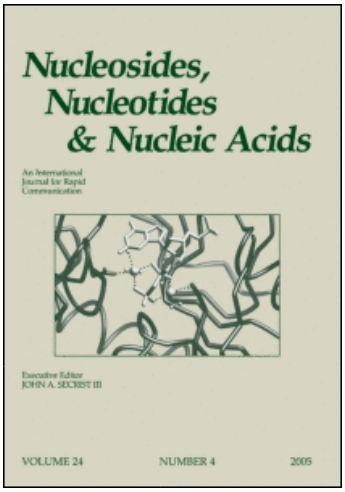

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

The Effect of pH on the Covalent Complexes of Thymidylate Synthase

Mary S. Weir[a]; R. Bruce Dunlap[a]
[a] Dept. of Chemistry and Biochemistry, University of South Carolina, Columbia, SC

To cite this Article Weir, Mary S. and Dunlap, R. Bruce(1994) 'The Effect of pH on the Covalent Complexes of Thymidylate Synthase', Nucleosides, Nucleotides and Nucleic Acids, 13: 1, 275 — 292
To link to this Article: DOI: 10.1080/15257779408013240
URL: http://dx.doi.org/10.1080/15257779408013240

NUCLEOSIDES & NUCLEOTIDES, 13(1-3), 275-292 (1994)

THE EFFECT OF PH ON THE COVALENT COMPLEXES OF THYMIDYLATE SYNTHASE

Mary S. Weir and R. Bruce Dunlap*
Dept. of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208

Abstract: The objective of this investigation was to study the effect of pH on the dissociation constants and binding ratios of covalent complexes of thymidylate synthases from *Escherichia coli* and *Lactobacillus casei.*

Introduction

Thymidylate synthase (TS) catalyzes the reductive methylation of 2'-deoxyuridine monophosphate (dUMP) to 2'-deoxythymidine monophosphate (dTMP) utilizing 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) as both reductant and source of the methyl group. This reaction is critically important in the cell as it provides the sole *de novo* source of dTMP for DNA synthesis. For this reason, the enzyme has been a target for chemotherapeutic antimetabolites, such as 5-fluoro-2'-deoxyuridine (FdU) and 10-propargyl-5,8,-dideazafolate (PDDF), both of which are inhibitors of TS.[1,2]

Most of the studies on the binary and ternary complexes of TS have utilized the enzyme isolated from amethopterin-resistant *Lactobacillus casei.*[3] The first evidence that TS could form a covalent binary complex with FdUMP was provided by ^{19}F-NMR.[4] These results were confirmed through a nitrocellulose filter technique and by a trichloroacetic acid precipitation procedure.[5,6] The presence of folates has been shown to increase both the extent and tightness of binding of nucleotides to the enzyme (for

This paper is dedicated to the memory of Dr. Roland K. Robins.

reviews, see references 7 and 8). The inhibitory ternary complex consisting of 5-fluorodeoxyuridine 5'-monophosphate (FdUMP), CH_2H_4folate, and TS has been studied by a number of techniques, such as gel electrophoresis, absorbance, fluorescence, and circular dichroic spectroscopy.[7-9] The ^{19}F NMR spectrum of the *L. casei* enzyme binary complex with FdUMP exhibited resonances at -1.5 ppm and -34.5 ppm, which represented noncovalently and covalently bound inhibitor,[10] respectively, while the ^{19}F spectrum of the inhibitory ternary complex revealed a single resonance at -12.5 ppm, which represented the product of a *trans* addition of cofactor and enzyme across the C-5, C-6 double bond of FdUMP.[11] More recently, Cisneros and Dunlap[12] have characterized the inhibitory ternary complex through a TCA precipitation procedure where it was shown that the presence of CH_2H_4folate could enhance the covalent binding of FdUMP to TS 2.5-fold over that seen for FdUMP alone.

In recent years, the easy isolation of *Escherichia coli TS* from recombinant bacterial sources has facilitated the solution of the x-ray crystal structures of a number of complexes of the enzyme, such as the ternary complex with dUMP and PDDF[13] and the inhibitory ternary complex with FdUMP, the biologically active form of FdU, and PDDF.[14] These crystal structures enable the investigator to identify the specific amino acid residues that play important roles in substrate binding and catalysis. It is possible to create site directed mutant forms of TS in which these amino acid residues are systematically altered in order to further study their action in the enzyme. However, it is crucial to understand the properties of the wild type enzyme first, in order to place the results with the various mutant enzymes in the proper context. Since the majority of the work on TS has been done on the *L. casei* enzyme and with much of the recent work on the *E. coli* TS, the comparison of the enzymes from *E. coli* and *L. casei* was a logical step in the pathway to study the site directed mutant enzymes.

The rate and extent of binding of substrates can be affected by many factors including temperature, time of incubation and the pH of the incubation buffer. By using the trichloroacetic acid precipitation assay, the effect of pH on the covalent binding of

the nucleotides, dUMP, FdUMP, and dTMP, to both *E. coli* and *L. casei TS,* both in the absence and the presence of folates has been investigated. In most cases the extent of binding was examined by determination of K_d through Scatchard analysis,[15] but due to the low binding ratio of some systems, the pH effect was examined by determination of maximum covalent binding ratio.

Materials and Methods: *Materials.* dUMP, dTMP, FdUMP, dithiothreitol, and 2-mercaptoethanol were purchased from Sigma. The radiolabeled nucleotides, [6-^{3}H]FdUMP, [6-^{3}H]dUMP, and [methyl-^{3}H]dTMP, were products of Moravek Biochemicals. The labeled dTMP is not contaminated with dUMP. Tris(hydroxymethyl)-aminomethane (Tris) and KCl were from Research Organics. PhastGel gradient gel 8-25%, PhastGel Blue R, PhastGel native buffer strips, Q-Sepharose fast flow and Phenyl Sepharose fast flow were purchased from Pharmacia. [6R]Tetrahydrofolate (H_4folate) was prepared by the enzymatic reduction of folic acid by *Lactobacillus casei* dihydrofolate reductase, and was converted to [6S]CH_2H_4folate by the addition of a 25-fold molar excess of formaldehyde in 125 mM $NaHCO_3$, pH 7.0, containing 300 mM 2-mercaptoethanol to form a stock cofactor solution (1 mM CH_2H_4folate). Folic acid was purchased from Calbiochem.

Purification of E. coli and L. casei Thymidylate Synthases. The *E. coli* cells were a gift of Agouron Pharmaceuticals. The purification scheme for both the *E. coli* and *L. casei* enzymes was similar to that described by Boles et al.[16] Separate suspensions of *E. coli* and *L. casei* cells were sonicated and the resulting cell free extracts were chromatographed successively on Q-Sepharose and Phenyl Sepharose columns. The chromatography was performed with a Pharmacia FPLC system equipped with LCC 500 liquid chromatography controller. All enzyme purification steps were performed at 5° C, and the buffers were degassed by bubbling with He gas for 30 min. The purified *E. coli* and *L. casei* enzymes exhibited specific activities of 7.4 and 2.5 units/mg, respectively, and were found to be greater than 95% homogeneous by native polyacrylamide gel electrophoresis.

Gel Electrophoretic Procedures. The purity of column fractions was assessed following electrophoresis of native and inhibitory ternary complexes of 20-fold concentrates of each column fraction exhibiting TS activity. Concentration of fraction aliquots was carried out with Centricon micro-concentrators (Centricon-30, Amicon Corp.). For native TS lanes, 1 μL of TS (1 mg/mL) was mixed with 2 μL of 0.1% bromophenol blue. Inhibitory ternary complexes were prepared for electrophoresis by incubating 1 μL of TS (1 mg/mL) with 1 μL 2.5 mM FdUMP and 1 μL of 1 mM CH_2H_4folate for 5 min. Samples were then subjected to electrophoresis on Pharmacia PhastGel Gradient Gel 8-25% utilizing PhastGel Native Buffer Strips with a Phast Electrophoresis System.

Protein Assays. The protein concentrations of column fractions and column pools were estimated by A_{280} values and further determined by the BioRad dye binding microassay using bovine serum albumin as the standard.[17] Concentrations of homogeneous TS were measured using the molar extinction coefficient of 127,000 M^{-1} cm^{-1} at 280 nm for the *E. coli* enzyme and 105,000 $M^{-1}cm^{-1}$ at 278 nm for the *L. casei* enzyme.[18]

Spectrophotometric Assay. Enzyme activity was determined by monitoring the increase in absorbance at 340 nm due to the formation of dihydrofolate for 30 sec after the addition of the enzyme to the substrate pre-mix.[3] Typically, 100 μL of a 2.0 μM TS stock solution was added to a 900 μL pre-mix solution consisting of 120 mM Tris, 60 mM MES, 60 mM acetic acid (Morrison buffer[19]), pH 7.0 containing 100 μM CH_2H_4folate and 100 μM dUMP. All spectrophotometric assays were carried out at 30°C. Data were collected with a Hewlett-Packard 8450A UV/VIS spectrophotometer and processed with an on-line computer.

Trichloroacetic Acid Precipitation Assay. The TCA assay was employed to determine maximum covalent FdUMP/TS binding ratios along with apparent K_d values for the inhibitory ternary complex formed from enzyme, FdUMP and CH_2H_4folate.[20] The 0.5 mL reaction mixture, containing 0.1 to 100 nM [6-^{3}H]FdUMP (24 Ci/mmole),

1 nM TS, 50 μM CH_2H_4folate and Morrison buffer, pH 7.0 was incubated at 37°C for 10 min followed by quenching with the addition of 125 μL of 50% TCA. This solution was then centrifuged at 14,000 rpm for 2 min, decanted, washed 4 times with 10% TCA and dissolved in an ethanolic wash (0.2 N NaOH in 50% ethanol) and transferred to scintillation vials for subsequent counting.

Binding ratios and K_d experiments. The covalent binary, pseudo ternary, and inhibitory ternary complex binding ratios and K_d values were determined with the TCA assay. Reaction mixtures (0.5 mL) containing 0.2 μM of enzyme with 50 μM [6-^{3}H]FdUMP for the binary complex and with 20 μM [6-^{3}H]FdUMP and 200 μM folate for the various ternary complexes and were incubated for 45 min at 37°C to obtain binding ratio data. In all measurements of binding ratios and K_d values, a Morrison buffer system was used to insure uniform ionic strength and constant pH values. Individual K_d experiments are described in the following paragraphs. Binding ratio and K_d experiments were performed three times and each TCA assay repeated in triplicate. Standard deviations of 10% or less were considered acceptable. The covalent binding ratio was defined as nmol FdUMP bound per nmol TS dimer.

The effect of pH on the K_d values for the covalent binary complex formed from FdUMP, dUMP, and dTMP with TS. TS (0.5 μM) was incubated for 45 min at 37°C with 100 μg/mL BSA and [6-^{3}H]FdUMP concentrations that varied from 5 to 100 μM. TS (0.5 μM) was incubated for 45 min at 37°C with 100 μg/mL BSA and [6-^{3}H]dUMP concentrations that varied from 4 to 200 μM. TS (1 μM) was incubated for 45 min at 37°C with 100 μg/mL BSA and 50 μM [methyl-^{3}H]dTMP. The reaction was quenched with 125 μL of 50% TCA. The precipitate was treated as described for the TCA precipitation assay.

The effect of folate on the binding of FdUMP to TS as a function of pH. E. coli TS (0.2 μM) was incubated for 15 min at 37°C with 20 μM [6-^{3}H]FdUMP, 100 μg/mL BSA and 100 μM CH_2H_4folate, H_4folate, or H_2folate. The reaction was quenched with 125 μL of 50% TCA and processed as described above. The binding ratio is the ratio of FdUMP bound to the enzyme dimer.

The effect of pH on the formation of the pseudo ternary complex of FdUMP, H_4folate and TS. *TS* (1 nM) was incubated for 15 min at 37°C with 20 μM [6R] H_4folate, 100 μg/mL BSA, 29 μM dimedon and [6-^{3}H]FdUMP concentration varying from 4 nM to 300 nM. The reaction was quenched with 125 μL of 50% TCA.

The effect of pH on the formation of the inhibitory ternary complex (ITC). Bacterial TS (1 nM) was incubated for 15 min at 37°C with 350 nM [6S]CH_2H_4folate, 100 μg/mL BSA, and a [6-^{3}H]FdUMP concentration ranging from 0.1 nM to 100 nM. Also in all cases the binding was determined by the TCA precipitation method[12] and K_d was determined by Scatchard analysis.[15]

Results: Thymidylate synthase can form covalent complexes with its nucleotide substrate, product, and inhibitor in both the presence and absence of folates.[21] Six sets of covalent complexes were studied as a function of pH, and these included three covalent nucleotide-enzyme binary complexes (enzyme with FdUMP, dUMP, or dTMP), two pseudo ternary complexes (enzyme with FdUMP and H_2folate or H_4folate), and the covalent inhibitory ternary complex of TS-FdUMP-CH_2H_4folate. Both maximum binding ratio, determined under saturating conditions of ligands, and dissociation constants, K_d, were measured for these covalent complexes.

To determine intrinsic dissociation constants, K_d, the binding site concentration should be 10-fold lower than the determined dissociation constant.[22] The K_d for FdUMP binding to *E. coli* TS is estimated to be 1.6 x 10^{-9} M.[23] Thus, determining the intrinsic K_d from FdUMP titration experiments would have required the use of low enzyme concentrations (below 8 x 10^{-11} M) and very high specific activity of [6-^{3}H]FdUMP. We found that binding experiments at the extreme pH values would have been prohibitively expensive under these conditions. Thus, we chose conditions that allowed us to easily determine the K_d values at the extreme pH values, and, therefore, the K_d values that we are determining must be termed apparent K_d values. In some cases, the maximum binding ratio was reported since the binding of substrate was too low to determine a K_d.

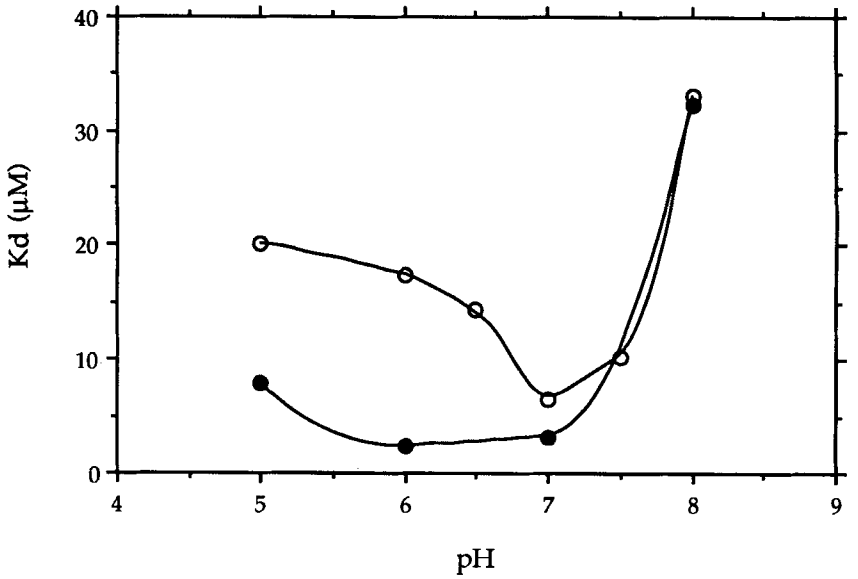

FIGURE 1: The effect of pH on the Kd for the covalent binary complex formed with FdUMP and TS. *E. coli TS (o)* and *L. casei TS (•)* (0.5 μM) TS was incubated for 45 min at 37°C with 100 μg/mL BSA and [6-^{3}H]FdUMP concentrations that varied from 5 to 100 μM.

The effect of pH on the K_d for the covalent binary complex formed from FdUMP and TS. Since FdUMP is a mechanism based inhibitor and binds to TS to a greater extent than dUMP, the effect of pH on the K_d on the binary complex formed with FdUMP was determined and found to be different for both bacterial enzymes (Figure 1 and Table 1). *L. casei* TS bound FdUMP to a greater extent at acidic pH values than at basic pH values. The K_d for the covalent binary complex reached a minimum at pH 6.0 and remained low over the pH range from 5 to 7. The K_d values dramatically increased at basic pH values, and it was not possible to determine the K_d for this complex above pH 8.0. The *E. coli* enzyme was also more likely to bind FdUMP at the lower pH values, but the K_d for this enzyme reached its minimum at pH 7.0. This K_d increased slightly at acidic pH values and increased dramatically at basic pH values.

TABLE 1: Maximum binding ratios and minimum K_d values of the complexes formed with *E. coli* and *L. casei* TS at pH 7.0.

Complex	*E. coli*		*L. casei*[a]	
	Binding ratio[b]	K_d	Binding ratio[b]	K_d
TS-FdUMP	0.6	6.5 μM	0.7	3.2 μM
TS-dUMP	0.2	3.7 μM	0.12	4.1 μM
TS-dTMP	0.02	[c]	0.18	[c]
TS-FdUMP--H_2folate	1.0	[c]	1.3	[c]
TS-FdUMP--H_4folate	1.7	3.8 nM	2.0	12.9 nM
TS-FdUMP-CH_2H_4folate	1.7	0.7 nM	1.7	1.2 nM

[a] Reference 21
[b] nmoles ligand/nmoles TS
[c] Not determined

The effect of pH on the K_d for the covalent binary complex formed from dUMP and TS. The latter differences in FdUMP binding found for the two bacterial enzymes raised questions concerning any differences in the binding of dUMP, the natural substrate, to the two enzymes. It was found that extent of binding of the nucleotide was greatly reduced from that seen for the FdUMP covalent binary complexes (Table 1). It was still possible to determine the pH dependence of K_d values for the covalent dUMP-TS binary complexes (Figure 2). The pH profile for each of the two bacterial enzymes for this complex was very similiar to the pH profile for the FdUMP binary complex at pH 7 and above. The *L. casei* enzyme exhibited a minimum K_d value for dUMP between pH 6 and 7. At pH 8 and 9 the K_d value increased between 4- and 5-fold, respectively. The *E. coli* enzyme exhibited a narrowed pH profile with minimum K_d value at pH 7 which increased 4.5-fold at pH 6 and 3.5-fold at pH 8.

The effect of pH on the covalent binding of dTMP to TS. In an effort to determine the extent of covalent binding of the nucleotide product to the *E. coli* enzyme, the binding ratio of dTMP was examined via the TCA assay as a function of pH (Figure 3). The maximum binding ratio (0.02) was achieved at pH 7.0, but the extent of covalent

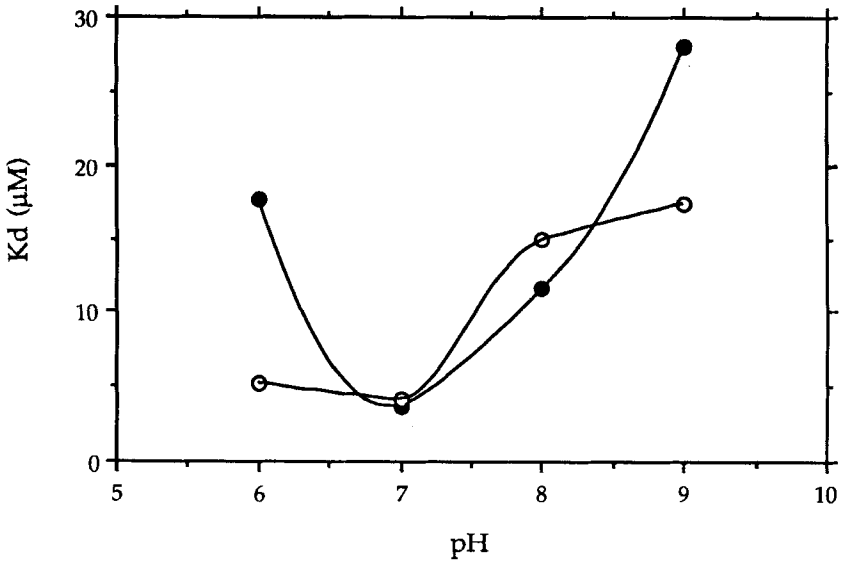

FIGURE 2: The effect of pH on the K_d for the covalent binary complex formed with dUMP and TS. *E. coli TS* (•)and *L. casei TS* (o) (0.5 μM) TS was incubated for 45 min at 37°C with 100 μg/mL BSA and [6-^{3}H]dUMP concentrations that varied from 4 to 200 μM.

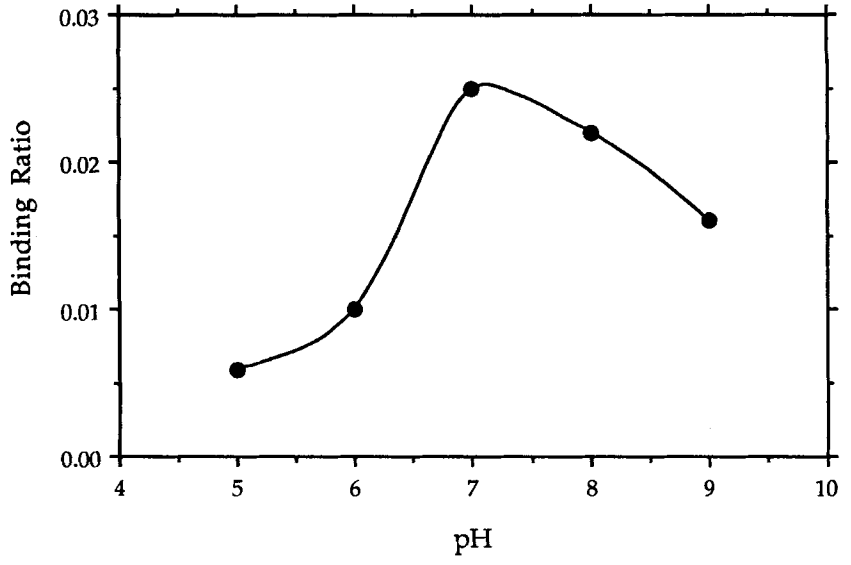

FIGURE 3: The effect of pH on the maximum binding of dTMP to *E. coli* TS. 1 μM TS was incubated for 45 min at 37°C with 100 μg/mL BSA and 50 μM [methyl-^{3}H]dTMP.

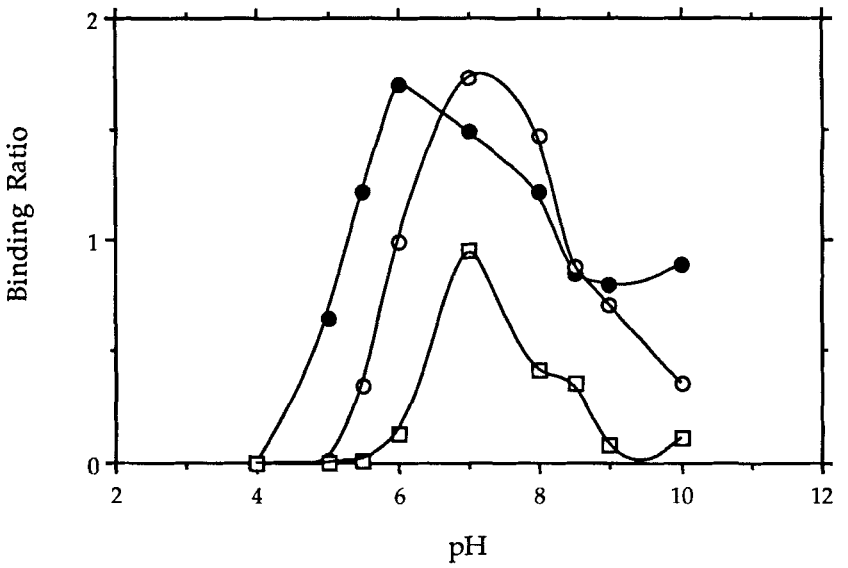

FIGURE 4: The effect of CH_2H_4folate, H_4folate, or H_2folate, on the binding of [6-^{3}H] FdUMP to TS as a function of pH. 0.2 μM *E. coli* TS was incubated for 15 min at 37°C with 20 μM [6-^{3}H]FdUMP, 100 μg/mL BSA and 100 μM CH_2H_4folate (•), H_4folate (o), and H_2folate (□). The binding ratio is the ratio of FdUMP bound to the enzyme dimer.

binding for dTMP to the *E. coli* enzyme was some 30-fold lower than the covalent binding ratio found for the FdUMP covalent binary complex. The covalent binding ratios for dTMP showed a relative stabilization under basic conditions, since they decreased some 12 and 40% at pH 8 and 9, respectively, as compared to a drop of 60% in covalent dTMP attachment at pH 6. The fact that dTMP formed a covalent bond to the *E. coli* TS to any extent was significant. These results suggested that the enzyme could catalyze at least the initial covalent catalysis step in the reverse reaction, although the activation energy of this reaction was clearly increased dramatically over that for the forward reaction. Another significant difference in nucleotide binding between the *L. casei* and the *E. coli* enzymes was the binding of dTMP which bound covalently to the *L. casei* enzyme (0.18) to a greater extent than dUMP (0.12). Covalent dTMP binding to the *E. coli* enzyme, on

the other hand, was severely limited (0.02) and was 10-fold lower than the maximum extent of dUMP binding.

The effect of folates on the covalent binding ratio of FdUMP to E. coli TS as a function of pH. It has been known for many years that folates can enhance nucleotide binding to TS.[10,24,25] Although a number of folates are present in the cell, only CH_2H_4folate can participate productively in the TS catalyzed reaction. The effect of pH on covalent FdUMP binding to the *E. coli* enzyme was examined for three covalent complexes, TS-FdUMP--H_2folate, TS-FdUMP--H_4folate, and TS-FdUMP-CH_2H_4folate (Figure 4). Saturating levels of H_2folate enhanced covalent FdUMP binding at pH 7.0, but only to a binding ratio of 1.0. These results are in accord with those of Lewis et al.[10] and Moore et al.[21], who showed that the presence of H_2folate shifts the FdUMP enzyme equilibrium in favor of covalently bound nucleotide. Significantly, the covalent binding ratio decreased by 50% at pH 8.0, but dropped almost 10-fold at pH 6.0.

The covalent pseudo ternary complex consisting of *E. coli* TS, FdUMP and H_4folate, exhibited a maximum binding ratio of 1.7 at pH 7.0. Besides nearly tripling the maximum binding ratio for the FdUMP binary complex, the presence of H_4folate significantly broadened (some 2 to 3-fold) the pH range for stability of this pseudo ternary complex (compare Figures 1 and 4). In the pH profile for the covalent inhibitory ternary complex, the maximum covalent binding ratio of 1.7 was achieved at pH 6, but the binding ratios at pH 7 and 8 were above 1.5 as well. The binding ratio at pH 6 (1.7) was nearly three times higher than that seen for the covalent binary complex (0.6) at the same pH. As the pH dropped below 6, the covalent binding ratio decreased dramatically, but at pH values at and above pH 8.3, the binding ratio stabilized at 1.0.

The maximum covalent nucleotide binding ratios in the binary and various ternary complexes determined for the *L. casei* enzyme[21] were compared to those found for *E. coli* TS (Table 1). FdUMP bound covalently to the *L. casei* enzyme (0.7) and to the *E. coli* enzyme (0.6) to roughly the same extent. H_2folate enhanced the binding of

FdUMP to the *L. casei* enzyme (1.3) to a greater extent than seen for the *E. coli* enzyme (1.0).

In the pseudo ternary complex with the *L. casei* enzyme, H_4folate promoted the covalent binding of FdUMP to all the available binding sites as indicated by the binding ratio of 2.0.[21] This is almost a 3-fold enhancement over that reported for the binary complex.[6] On the other hand, when H_4folate enhanced the covalent binding of FdUMP nearly 3-fold over that seen for the binary complex for the *E. coli* enzyme, the maximum binding ratio was 1.7. Similar maximum binding ratios were found for the inhibitory ternary complexes for both the *L. casei* and *E. coli* enzymes. It is not clear why H_4folate should have induced covalent binding to all the binding sites in the *L. casei* enzyme while CH_2H_4folate, the natural substrate, should only enhance the binding of FdUMP to a binding ratio of 1.7 with the enzyme.

The effect of pH on the K_d of ternary complexes of FdUMP and TS with H_4folate or CH_2H_4folate. As shown in Table 1, the presence of saturating levels of H_4folate, produced a substantial cooperativity effect in stimulating covalent FdUMP binding, even when the 200-fold difference in enzyme concentration was considered (see Materials and Methods). When comparing the pH dependence of K_d for the FdUMP binary complex (Figure 1) with that for the pseudo ternary complex of FdUMP,TS, and H_4folate (Figure 5), the most striking feature was that K_d for the latter pseudo ternary complex remained at or near its minimum value over at least 2 orders of magnitude in $[H^+]$ (pH 6 to 8)for the *L. casei* enzyme and over 3.5 orders of magnitude in $[H^+]$ (pH 5.5 to 9) for the *E. coli* enzyme.

As shown in Table 1, the K_d values for the inhibitory ternary complexes for the *E. coli* and *L. casei* enzymes were 0.7 nM and 1.2 nM, respectively, which represented 5.4- and 10.8-fold decreases from the pseudo ternary complexes for the same enzymes. The increase in the tightness of binding can be attributed to the fact that CH_2H_4folate forms a covalent bridge with FdUMP whereas H_4folate cannot. As with the pseudo ternary complex, both of the enzymes were able to form the inhibitory ternary complex

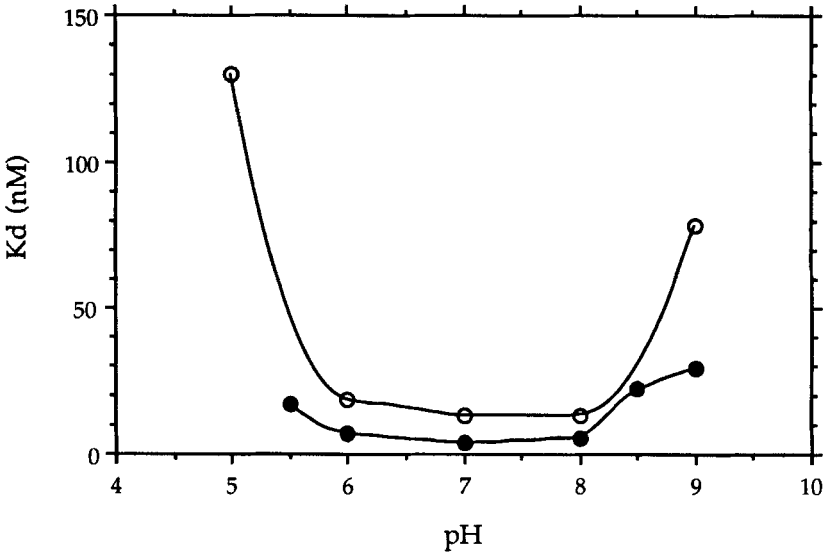

FIGURE 5: The effect of pH on the K_d for the covalent pseudo ternary complex formed with FdUMP, H_4folate, and TS. *E. coli TS (•)* and *L. casei TS (o)* (1 μM) was incubated for 15 min at 37°C with 100 μg/mL BSA, 20 μM H_4folate and [6-^{3}H] FdUMP concentrations that varied from 4 to 300 nM.

in the presence of CH_2H_4folate over a large pH range (Figure 6), but the *E. coli* enzyme was more resistant to the effects of basic pH values than the *L. casei* enzyme. It was clear that the inhibitory ternary complexes of both bacterial enzymes were destabilized below pH 5, and thus the K_d's could not be determined below pH 5 due to the low extent of binding of nucleotide to the enzyme.

One major difference between the two bacterial enzymes was the consistently wider pH range in which the *E. coli* enzyme could bind FdUMP covalently in the presence of folates as compared to the *L. casei* enzyme. In comparing the pH range of minimum K_d values for the covalent FdUMP binary complex and inhibitory ternary complex (Figures 1 and 6) it is remarkable that the inhibitory ternary complex was stable over a 5,000-fold range of $[H^+]$, as compared to the 100-fold $[H^+]$ range for the binary complex.

Discussion: The interaction of nucleotides, especially FdUMP, dUMP, and dTMP, in the presence and absence of folates with thymidylate synthase, has been studied

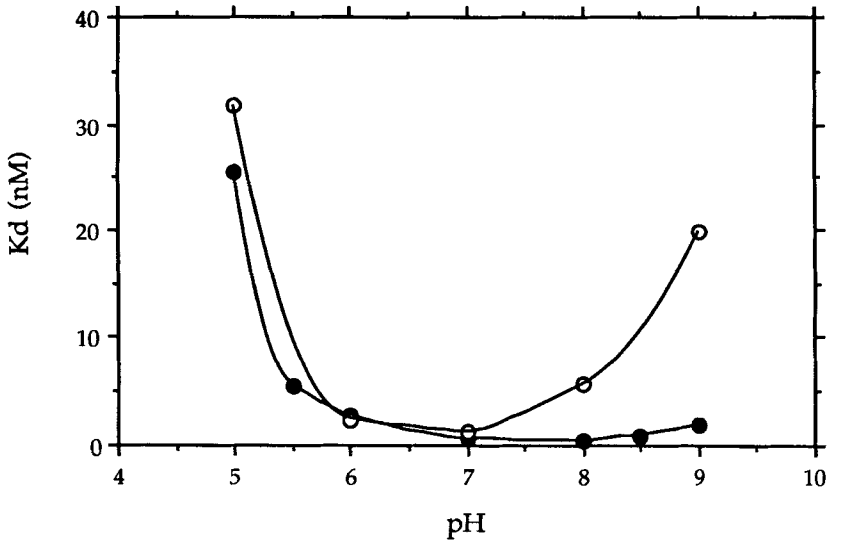

FIGURE 6: The effect of pH on the K_d for the covalent inhibitory ternary complex formed with FdUMP, CH_2H_4folate, and TS. *E. coli TS* (•) and *L. casei TS* (o) (μM) was incubated for 15 min at 37°C with 100 μg/mL BSA, 350 nM CH_2H_4folate and [6-^{3}H]FdUMP concentrations that varied from 0.1 to 100 nM.

by methods such as ^{19}F NMR,[10,11] nitrocellulose filtration,[5] and the trichloroacetic acid precipitation assay.[6] The latter technique was especially useful in demonstrating that FdUMP, dUMP, and dTMP each formed covalent binary complexes with *L. casei* TS and that the presence of folates increased the maximum binding stoichiometry of FdUMP and dUMP. The nucleotides, FdUMP, dUMP, and dTMP can form binary covalent complexes with TS and covalent ternary complexes in the presence of folates. However, these studies were usually conducted at only a single pH value. The objective of the present study was to evaluate and compare the apparent dissociation constants, K_d, and the maximum covalent binding ratios as a function of pH for FdUMP, dUMP, and dTMP interaction with *E. coli* and *L. casei TS* in the presence and absence of added folates. In most cases, the complexes were most stable at neutral pH values. A few complexes, though, exhibited a preference for acidic or basic pH values. It was not clear whether

the instability of certain complexes at the extreme pH's was due to the titration of the side chains of amino acid residues important for binding and for catalysis, or the titration of pH sensitive functional groups of the ligands.

The apparent dissociation constants were reported since the "true" dissociation constants were those reported at the lowest enzyme concentrations possible. Due to the range of specific radioactivity available for the nucleotides, the lowest enzyme concentration that was possible to study was 1 nM.

Both bacterial enzymes exhibited similiar K_d versus pH profiles for the covalent binding of FdUMP and dUMP in the binary complexes. For example, at pH 7, the K_d values for FdUMP and dUMP binding were between 3 and 8 μM for TS from both bacterial sources and were thus about 1000 times greater than the K_d values for the inhibitory ternary complex, and, they agree with those values reported by Galivan et al.[26] As observed previously with the *L. casei* enzyme,[6] dTMP was also capable of forming covalent binary complexes with the *E. coli* enzyme. However, the extent of covalent binding was quite low (0.02 dTMP/enzyme dimer) and was maximal at pH 7. The fact that dTMP could bind covalently to both *E. coli* and *L. casei TS* was surprising and may indicate the possibility of one or two steps of the reverse reaction proceeding at a much slower rate, though the *L. casei* enzyme was a better candidate for this reaction due to the greater affinity of dTMP and H_2folate for this enzyme.

In general, both bacterial enzymes yielded K_d versus pH profiles which were similar to one another, whether for the covalent inhibitory ternary complex formation, the covalent pseudo ternary complex formation of FdUMP, enzyme and H_4folate, or the covalent binary complexes with FdUMP or dUMP. The most remarkable feature of the K_d versus pH plot for the inhibitory ternary complex was the fact that the value for K_d changed less than 10-fold over the pH range from 5.5 to 9.0. A similar span for K_d of the inhibitory ternary complex was reported by Zhang et al.[26] with recombinant mouse TS. Viewed in another way, these results reflect the fact that as the enzyme forms successively tighter complexes with FdUMP and various folate ligands, its active site

regions become increasingly insulated from changes in pH of the bulk solvent. These observations are completely consistent with the differences in the x-ray crystal structure of the apoenzyme [28] and those for various binary and ternary complexes which indicated that complex formation, especially associated with folate binding, is accompanied by major conformation changes which compact and tighten the protein structure.[13,14,29] Furthermore, our findings and interpretations are in accord with the substantial decreases in protein volume of some 3.25%, which were initially measured in gel filtration and ultracentrifugation studies conducted by Lockshin and Danenberg[30] and confirmed by us (S. R. Southworth and R. B. Dunlap, unpublished results) experienced on converting the apoenzyme to the inhibitory ternary complex.

Acknowledgements: Support for this work was provided by National Institutes of Health grant CA 15645 from the National Cancer Institute. We thank Dr. Ernie Villafranca and Agouron Pharmaceuticals for the *E. coli* cells and their support.

REFERENCES

1. Duschinsky, R., Pleven, E. and Heidelberger, C., *J. Am. Chem. Soc.* **1959**, 79, 4559-4560 .
2. Jones, T. R., *Eur. J. Cancer* **1980**, 16, 707-711.
3. Dunlap, R B., Harding N. G. L. and Huennekens, F. M., *Biochemistry* **1971**, 10, 88-97.
4. Lewis, C. A., Ellis, P. D. and Dunlap, R. B., *Biochemistry* **1980**, 19,116-123.
5. Ahmed, F., Moore, M.A. and Dunlap, R.B., *Anal. Biochem.* **1985**, 145,151-159.
6. Moore, M. A., Ahmed, F. and Dunlap, R. B., *Biochem. Biophys. Res. Commun.* **1984**, 124, 37-43.
7. Lewis, C. A. and Dunlap, R. B., in *Topics in Molecular Pharmacology* (Burgen, A. 5. V. & Roberts, eds) pp. 169-219, Elsevier/North Holland Biomedical Press, Amsterdam, 1981b.

8. Santi, D.V. and Danenberg, P.V. in Folates and Pterins (Blakley, R.L. and Benkovic, S.J. eds) pp. 345-398, John Wiley & Sons, New York, 1984.

9. Donato, H., Aull, J. L., Lyon, J. A., Reinsch, J. W. and Dunlap, R. B., *J. Biol. Chem.* **1976**, 254, 1303-1310.

10. Lewis, C A., Ellis, P. D. and Dunlap, R. B., *Biochemistry* **1981**, 20, 2275-2285.

11. Byrd, R. A., Dawson, W. H., Ellis, P. D. and Dunlap, R. B., *J. Am. Chem. Soc.* **1978**, 99, 6139-6141.

12. Cisneros, R. J. and Dunlap, R. B., *Biochem. Biophys. Acta.* **1990**, 1039, 149-156.

13. Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G.F., Hardy, L., Maley, F. and Stroud, R.M., *Biochemistry* **1990**, 29, 6964-6977.

14. Matthews, D. A., Appelt, K. and Oatley, S. J., *J. Mol. Biol.* **1989**, 205, 449-454.

15. Scatchard, G. Ann. N. Y., *Acad. Sci.* **1949**, 51, 660-672.

16. Boles, J. 0., Cisneros, R. J., Weir, M. 5., Odom, J. D., Villafranca, J. E. and Dunlap, R. B., *Biochemistry* **1991**, 30,11073-11080.

17. Bradford, M., *Anal. Biochem.* **1976**, 72, 248-254.

18. Lyon, J. A., Pollard, A. P., Loeble, R. B. and Dunlap, R. B., *Cancer Biochem. Biophys.* **1975**, 1,121-128.

19. Ellis, K. J., & Morrison, J. F., *Methods Enzymol.* **1982**, 87, 405-426.

20. Cisneros, R. J. and Dunlap, R. B., *Anal. Biochem.* **1990**, 186, 202-208.

21. Moore, M. A., Ahmed, F. and Dunlap, R. B., *J. Biol. Chem.* **1986**, 261,12749-12749.

22. Connors, K. A., in: *Binding Constants*; John Wiley: NY, 1987.

23. Dev, I., Yates, B. B., Leong, J. and Dallas, W. S., *Proc. Natl. Acad. Sci. U. S. A.* **1988**, 83,1472-1476.

24. Santi, D. V., McHenry, C. S., Raines, R. T. and Ivanetich, K. M., *Biochemistry* **1977**, 26, 8606-8613.

25. Lockshin, A. and Danenberg, P.V., *Biochem. Pharm.* **1981**, 30, 247-257.

26. Zhang, H., Cisneros, R J., Deng, W., Zapf, J. W., Johnson, L. F. and Dunlap, R. B., *Biochem. Biophys. Acta.* **1991**, 1077, 35-46.

27. Galivan, J.H., Maley, G.F. and Maley, F., *Biochemistry* **1976**, 15, 356-362.

28. Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. 0., Santi, D. V. and Stroud, R. M., *Science* **1987**, 235, 448-455.

29. Kamb, A., Finer-Moore, J. S. and Stroud, R. M., *Biochemistry* **1992**, 31,12876-12884.

30. Lockshin, A. and Danenberg, P. V., *Biochemistry* **1980**, 19, 4244-4251.

Received 8/2/93
Accepted 9/21/93