Thermodynamic analysis of the binding of 5-fluoro-2′-deoxyuridine 5′-monophosphate to thymidylate synthase over a range of temperatures

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The binding of 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) to *Lactobacillus casei* recombinant thymidylate synthase has been studied by isothermal titration microcalorimetry at pH 7.1 over the temperature range 16−35 °C. Calorimetric measurements in various buffer systems with different heats of ionization suggest that a proton uptake is involved in the binding process of the nucleotide. In the temperature range investigated, the mol protons bound/mol nucleotide increases as the temperature decreases.

A model of two equal and independent sites fits well with the binding isotherms for thymidylate synthase. The binding constants, the changes in Gibbs energy, enthalpy, and entropy/site for FdUMP binding were calculated at each temperature. The results show that the binding is driven by both enthalpy and entropy contributions in the range 16−35 °C. The enthalpy changes become more negative as the temperature increases, with $\Delta C_p = -170 \pm 20\ J \cdot K^{-1} \cdot (\text{mol FdUMP bound})^{-1}$. The behavior of the system supports the observation that FdUMP binds to thymidylate synthase without producing profound conformational changes in the protein dimer.

Keywords: thymidylate synthase; 5-fluoro-2′-deoxyuridine 5′-monophosphate; microcalorimetry, binding.

Thymidylate synthase (TS) catalyzes the reductive methylation of deoxyuridine 5′-monophosphate (dUMP) to produce thymidine 5′-monophosphate (dTMP), with the cofactor 5,10-methylenetetrahydrofolate (CH_2H_4folate) being converted to dihydrofolate (H_2folate). The synthesis of thymidine 5′-monophosphate is essential for the biosynthesis of DNA [1, 2] and TS has received much attention as a target for anticancer drugs. One of the compounds most widely used is 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), a potent mechanism-based inhibitor of TS. The inhibition is known to result from the formation of a very tight, albeit reversible, covalent TS-FdUMP-CH_2H_4folate complex [3].

In *Lactobacillus casei*, the enzyme is a dimer of identical subunits of 36 kDa, each of which contains an active site. The crystal structure of TS bound to dUMP has been recently solved at 0.255 nm resolution [4]. The binary complexes are symmetric dimers with dUMP bound equivalently and noncovalently in both active sites [4], basically in the same conformation as in the *Escherichia coli* TS ternary complexes [5, 6]. Binding of FdUMP to TS has been studied by equilibrium dialysis [7], circular dichroism [8], and microcalorimetry [9, 10]. Equilibrium dialysis experiments in 50 mM Tris/HCl, pH 7.1, have revealed two binding sites in the dimeric enzyme for the inhibitor [7]. In contrast, Beaudette et al. have interpreted their calorimetric data on the basis of a single-site model [10].

Using isothermal titration calorimetry, we have studied the binding of FdUMP to TS at 16, 25, and 35 °C. The characterization of the binding was carried out at pH 7.1 in a series of buffers with different heats of ionization. Our data are consistent with a non-cooperative binding to two equal sites and we have calculated the thermodynamic functions over a range of temperatures. We have made some observations about the possible interactions responsible for these thermodynamic parameters.

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Abbreviations. TS, thymidylate synthase; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylenetetrahydrofolate; H_2folate, dihydrofolate.

Enzyme. Thymidylate synthase (EC 2.1.1.45).

MATERIALS AND METHODS

Chemicals. 1,4-Piperazinediethanesulfonic acid (Pipes), tris(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), ethylenediaminetetraacetic acid (EDTA), and 2-mercaptoethanol were purchased from Merck. 5-Fluoro-2′-deoxyuridine 5′-monophosphate, 2′-deoxyuridine 5′-monophosphate, and CH_2H_4folate were from Sigma. Sephadex G-25 was purchased from Pharmacia. Centriprep 30 concentrators were from Amicon. All chemicals used were of the highest purity available.

Enzyme. TS was purified from the Thy$^-$ *E. coli* strain χ2913 transformed with pKPTSd, which contains the complete coding sequence for the TS of *L. casei* [11]. Purification involved se-

quential chromatography on phosphocellulose and hydroxylapatite as described by Kealey and Santi [12].

After purification, TS was concentrated to 20–25 mg/ml and the buffer changed to 10 mM KH_2PO_4, pH 6.8, 0.1 mM EDTA. The enzyme showed a single-band pattern in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and purified protein was stored at −80 °C. The purification yield was approximately 100 mg apparently pure protein/l culture.

TS solutions were concentrated on Centriprep 30 concentrators and passed through a Sephadex G-25 column equilibrated with 50 mM KCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM buffer (Pipes, Tris, Hepes), adjusted to pH 7.1. The protein concentration was determined from absorption measurements at 278 nm, using the absorption coefficient of $1.256 \times 10^5\ M^{-1} \cdot cm^{-1}$ [13]. The activity of thymidylate synthase was measured spectrophotometrically by monitoring the increase in absorbance at 340 nm as described by Pogolotti et al. [14]. Reaction mixtures contained 0.1 mM CH_2H_4folate, 0.125 mM dUMP, and 10–14 nM protein. A unit of thymidylate synthase activity is defined as the amount necessary to synthesize 1 µmol dTMP/min at 25 °C. Specific activities of the purified protein were 2.5–3 U/mg.

The concentration of FdUMP was determined from absorbance measurements at 265 nm using a molar absorption coefficient of $8.08 \times 10^3\ M^{-1} \cdot cm^{-1}$ [15]. Absorbance measurements were carried out in a Beckman DU-70 spectrophotometer with the cells maintained at 25 °C.

Techniques. The equilibrium dialysis experiments were performed at 25 °C using a Dianorm equilibrium dialysis system with Spectrapor 12–14-kDa molecular mass cut-off membranes and a Beckman DU-70 spectrophotometer with a cuvette of 0.2-cm pathlength. The enzyme and ligand samples (500 µl each) were added to opposite sides of the dialysis membrane with a Hamilton microsyringe. The dimeric enzyme and FdUMP concentrations were 97.44 µM and 1 mM, respectively, both in 50 mM Tris/HCl, 50 mM KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.1. After 3 h dialysis, the FdUMP concentration was measured in the compartment without protein. Thus, the concentration of free ligand was calculated. The concentration of bound FdUMP was determined from the difference between the FdUMP concentration placed in a chamber (1 mM) and two times the FdUMP concentration measured in the side without protein. Controls, with ligand alone, revealed that dialysis equilibrium had been attained, and with enzyme alone, showed no loss in activity during dialysis. A duplicate of the experiment agreed within 3%.

For the calorimetric experiments, we have made use of an isothermal titration calorimeter designed, built, and optimized in our laboratory. The sensitivity of this instrument is enhanced by the heat compensation technique [16, 17] for which we have developed a theoretical study applying basic ideas of response theory. Heat compensation is carried out by a negative feedback control system that modifies the overall transfer function of the calorimeter. The instrument response is faster improving the signal-to-noise ratio and the stability of the calorimetric signal (this analysis and the implementation of this calorimeter will also be published). Electrical and chemical calibrations of the calorimeter response were performed in the same range as those obtained in the calorimetric experiments. Chemical calibration was accomplished by the neutralization of glycine with HCl [18]. Experiments were initiated by filling the sample and reference cells with 2.9 ml thymidylate synthase and buffer, respectively. After some time under temperature control, all components of the instrument reach thermal equilibrium to the desired temperature. The enzyme solutions and buffers were thoroughly degassed before being loaded into the calorimeter cells. Identical volumes of reactant and buffer are then injected into the sample titration and reference cells, respectively. Injection volumes were 20 µl or 30 µl and an equilibration time of 7 min was allowed between each injection. Dual injection compensates for most of the heat effects from non-chemical reaction sources. The heat effect arising from dilution of the titrant was measured in a separate experiment where the titration and reference cells were filled with buffer solution. These contributions to the observed heats of reaction are substracted from the corresponding total heats. The heat effect of the enzyme dilution was negligible in all cases. TS activity was routinely checked just before and after the titration experiment. Likewise, the pH values of the buffer, FdUMP, and protein solutions were checked at 16, 25 or 35 °C before and after the binding reaction.

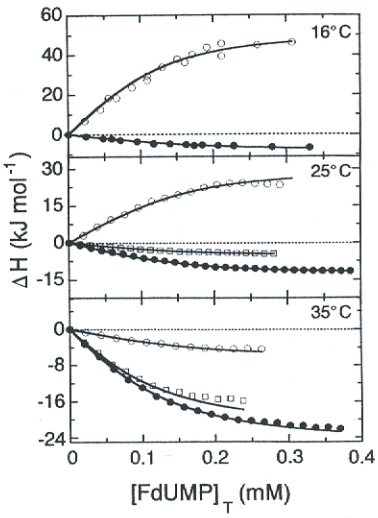

Fig. 1. Calorimetric titration of *L. casei* recombinant thymidylate synthase with FdUMP at pH 7.1 and 16, 25, and 35 °C. The dimeric protein concentration was in the range 0.06–0.1 mM in 50 mM KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol. The buffers used were: Tris/HCl (○); 50 mM Hepes (□); 50 mM Pipes (●). The data points show the total cumulative heat effect during the titration and the solid curves are the theoretical ones corresponding to Eqn (6) and obtained from the values shown in Table 1 using the values for the number of protons given in the text.

RESULTS

The calorimetric titrations of TS with FdUMP, in three different buffers at pH 7.1 and at 16, 25, and 35 °C, are shown (Fig. 1). The buffer systems employed were 50 mM Hepes, 50 mM Pipes, 50 mM Tris/HCl, containing 50 mM KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol. Due to the compensation between dilution of ligand, ionization or protonation of buffer and binding heats, the titration did not display any detectable peak in Hepes at 16 °C. The binding of FdUMP was exothermic in Pipes at the three temperatures investigated, while it was endothermic in Tris/HCl at 16 °C and 25 °C, and exothermic at 35 °C. Therefore, as a result of inhibitor binding, the state of protonation of the protein changed and the extent of this change depended on the temperature. As the enthalpy changes in Pipes buffer are more exothermic than those in Hepes or in Tris/HCl buffers, it followed that protons were taken up by the ligand-protein complex. Therefore the analysis of the calorimetric curves requires that the heat of ionization of the buffers should be known at each temperature; these values have been reported in the literature [18].

Table 1. Apparent thermodynamic parameters for the binding of one molecule of FdUMP to *L. casei* recombinant thymidylate synthase at pH 7.1. The uncertainties are standard errors in the fitting of the curves.

Temperature	$K \times 10^{-4}$	ΔG^0	ΔH_b	ΔS^0
	M^{-1}	kJ · mol^{-1}	kJ · mol^{-1}	J · K^{-1} · mol^{-1}
16	3.4 ± 0.8	−25.1 ± 0.6	−13.4 ± 1.2	40.2 ± 2.0
25	2.8 ± 0.7	−25.4 ± 0.7	−14.6 ± 1.6	36.3 ± 2.2
35	2.6 ± 0.5	−26.1 ± 0.5	−16.7 ± 1.9	30.4 ± 1.6

Previous equilibrium dialysis studies have shown that 2 mol FdUMP were bound/mol TS dimer in 50 mM Tris/HCl, while calorimetric data have been analyzed assuming a single site [10]. Our calorimetric curves showed that the enzyme is saturated at 0.5 mM total FdUMP. Thus, binding of FdUMP to the thymidylate synthase dimer was observed by equilibrium dialysis at this total concentration of inhibitor (see Materials and Methods section). At the equilibrium, the free ligand concentration was 0.4 mM, and 2 mol FdUMP was bound/mol dimeric enzyme.

All calorimetric titration curves were hyperbolic (non-cooperative), and the analysis of the calorimetric data was consistent with a binding process of FdUMP to two equal and independent sites. Hence, for each injection, the heat released or absorbed, is given by:

$$Q = mV[P]\Delta H_i \Delta Y, \tag{1}$$

where m stands for the number of binding sites, Q and ΔY for the heat and the increase in the saturation fraction associated with the change in bound ligand concentration, $[P]$ for the dimer protein concentration in the calorimeter cell, V for the reaction volume, and ΔH_i for the corresponding total heat/mol bound ligand, which can itself be expressed as

$$\Delta H_i = \Delta H_b + n\Delta H_i, \tag{2}$$

with n being the number of protons exchanged between the protein-ligand complex and the buffer during binding, ΔH_i the heat of buffer ionization, and ΔH_b the enthalpy change/mol FdUMP bound in a hypothetical buffer with zero ionization heat. Since the sample volume, V, increases after each injection, the protein concentration, $[P]$, decreases throughout the titration, however the number of mol protein ($[P] \cdot V$) is always kept constant.

Eqn (1) can be expressed as a function of the apparent microscopic association constant, K, and the free ligand concentration before and after each injection:

$$Q = mV[P]\Delta H_i \left(\frac{K[\text{FdUMP}]_j}{1 + K[\text{FdUMP}]_j} - \frac{K[\text{FdUMP}]_{j-1}}{1 + K[\text{FdUMP}]_{j-1}} \right) \tag{3}$$

The thermodynamic binding parameters at each temperature and for each buffer have been obtained by fitting the thermal data to Eqn (3). When the value of m in this equation was allowed to vary as one of the parameters of the fit during data analysis, final m values were 1.7−2.1. We are assuming, according to our equilibrium dialysis results, a value of $m = 2$ for the number of binding sites and we have used the nonlinear least-squares simplex algorithm [19]. In the calorimeter experiments, only the total ligand concentration, $[\text{FdUMP}]_T$, is known. The free ligand concentration after each injection must be calculated in each step of the optimization process from the total ligand and protein concentrations according to:

$$[\text{FdUMP}] = \{\sqrt{(1 - K[\text{FdUMP}]_T + mK[P])^2 + 4K[\text{FdUMP}]_T} + K[\text{FdUMP}]_T - mK[P] - 1\}/2K. \tag{4}$$

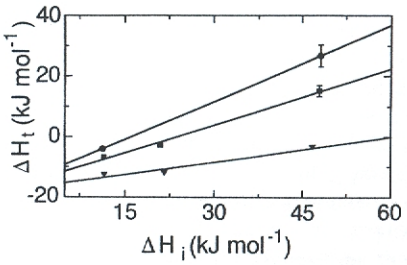

Fig. 2. Enthalpy change of binding, ΔH_t, of FdUMP to *L. casei* recombinant thymidylate synthase monomer as a function of the heat of ionization, ΔH_i, of the buffer. The solid lines represent the linear least-squares fit of the data obtained at 16 (●), 25 °C (■) and 35 °C (▼). The binding enthalpy changes in a hypothetical buffer with zero ionization heat, given by the intercept, are shown in Table 1.

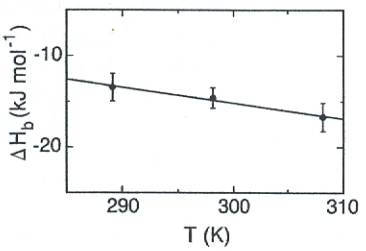

Fig. 3. Temperature dependence of the enthalpy change for the binding of FdUMP to *L. casei* recombinant thymidylate synthase at pH 7.1. The data are given in Table 1. The *solid line* is the linear least-squares fit of the data. The heat capacity change, given by the slope, is -170 ± 20 J · K^{-1} · mol^{-1}.

Once the convergence criteria are satisfied, the ΔH_t and K values are obtained for each buffer. At each temperature, the obtained K values are almost equal for the three different buffers and the averaged value is taken as the best estimation for K. However, ΔH_b and n are calculated from ΔH_t for each buffer using Eqn (2) (Fig. 2). The numbers of protons taken up by the TS subunit during FdUMP binding are 0.84 ± 0.04, 0.62 ± 0.05, and 0.28 ± 0.06 for 16, 25, and 35 °C, respectively. The variation of ΔH_b versus T is shown in Fig. 3; the slope provides the ΔC_p value -170 ± 20 J · K^{-1} · (mol FdUMP bound)$^{-1}$. In the temperature range studied, ΔC_p is independent of temperature. Gibbs energy and entropy changes for the binding of FdUMP were obtained from the microscopic binding constants and enthalpy changes at each temperature, and these functions are displayed in Table 1. The standard state is that of 1 mol l^{-1}. The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to the observed ones.

The equilibrium constants at the three temperatures studied should be initially consistent in terms of the van't Hoff equation. Thus, the K values at 16 °C and 35 °C can be recalculated from the value at 25 °C using the ΔH_b and ΔC_p values obtained. The integrated form of the van't Hoff equation is

$$\ln\left[\frac{K(T_2)}{K(T_1)}\right] = \frac{1}{R}\left(\frac{\Delta H_b(T_1)}{T_1} - \frac{\Delta H_b(T_2)}{T_2}\right) + \frac{\Delta C_p}{R}\ln\frac{T_2}{T_1}. \tag{5}$$

Minor variations arose in the constant values thus obtained from Eqn (5) at 16 °C and 35 °C.

The cumulative amount of heat released or absorbed as a result of bound ligand is directly proportional to the saturation fraction, Y, according to the equation

$$\Delta H = m(\Delta H_b + n\Delta H_i)\frac{K[\text{FdUMP}]}{1 + K[\text{FdUMP}]}. \quad (6)$$

Fig. 1 displays the binding curves in terms of cumulated heat for FdUMP-thymidylate synthase. Hence, any point, at a given ligand concentration, represents the total heat obtained summing all previous heats. The solid lines are those corresponding to Eqn (6), where the free concentration of FdUMP is given by Eqn (4), for the thermodynamic parameters shown in Table 1.

DISCUSSION

The formation of the binary complex of TS with FdUMP has been demonstrated by equilibrium dialysis [7], circular dichroism [8], and microcalorimetry [9, 10]. While equilibrium dialysis experiments showed that the two binding sites for FdUMP are equivalent [7], there is information suggesting that binding of FdUMP to one active site induces an asymmetry in the dimer that prevents binding to the second site [10, 20].

There is evidence indicating the existence of covalent binary enzyme-ligand adducts. Pogolotti et al. [21] showed that a very slow enzyme-catalyzed H5 exchange occurred in the absence of cofactor. Likewise, NMR studies [22] with native enzyme indicate that both the covalent and noncovalent enzyme-FdUMP binary complexes could form in the absence of CH_2H_4folate. While equilibrium dialysis studies have shown that TS binds 2 mol FdUMP/dimer, the trichloroacetic acid precipitation assay, which permits the direct quantitation of covalent complexes, indicated that the amount of nucleotide bound covalently to the enzyme was 0.7 mol [23].

However, the results obtained in this study could be fitted to a model of two equal and independent sites for FdUMP. The apparent binding constant value obtained at 25°C agrees with that published for equilibrium dialysis [7]. The equivalent and the noncooperative character of the binding sites displayed by the system at 25°C seem to be conserved at 16°C and 35°C. At these temperatures, neither information has previously been reported. The association constant values obtained from the analysis of calorimetric curves at these temperatures agree, within experimental errors, with the K values obtained from the van't Hoff equation using the ΔH_b and ΔC_p values given in Table 1. The affinity of FdUMP for TS seems to be practically the same throught the 16–35°C temperature range (Table 1). This enthalpy-entropy compensation is frequent in both noncooperative [24] and cooperative binding processes [25]. Also, it is the main thermodynamic characteristic of the marginal stability of the native conformation in proteins and other biopolymers [26, 27].

In order to analyze the calorimetric data correctly, the first question that needs to be answered is whether there is any proton exchange during binding. Thus, we have carried out the thermal titration in various buffers of sufficiently different ionization heats. The heats obtained when TS becomes saturated with FdUMP in Tris/HCl or Hepes buffers at 25°C are similar to the corresponding values obtained by Beaudette et al. [9, 10]. These authors interpreted their data first in terms of two non-equal independent sites [9] and in a later study [10] as a model that assumes a single binding site. This interpretation contrasts with the crystallographic data indicating that the enzyme is a dimer of identical subunits [28] and that dUMP binds to TS in an equivalent manner to both active sites [4]. By equilibrium dialysis we have obtained 2 mol FdUMP bound/mol enzyme at saturation conditions for the ligand. A model that assumes two identical independent sites is also in good agreement with the calorimetric curves obtained using different buffers and temperatures.

From our experiments in different buffers, we conclude that there is a net proton uptake on behalf of the FdUMP-thymidylate synthase complex during the binding process. This means that one or several pK values, corresponding to some proton accepting groups of the nucleotide and/or enzyme, increase (i.e. become less acidic). Since 0.8 H^+/enzyme monomer are taken up at 16°C, probably more than one ionizable group changes its pK value. Alterations in the protonation state of certain residues in the vicinity of the FdUMP-binding site may explain the uptake of protons when FdUMP binds to the enzyme at pH 7.1. Crystallographic studies have shown that there are two histidine residues in the active site of the TS [4, 28]; a change in the pK value of this residue may explain the uptake of protons. However, other groups of the ligand and/or protein may contribute in the protonation of complex and this cannot be clarified using our calorimetric study. There are several reasons that may explain these changes in pK values. For instance, a variation in the micropolarity of the environment surrounding the side chains of certain active site residues as a result of FdUMP binding is a possibility. Alternatively, a protonated form could be stabilized by forming a hydrogen bond with a neighboring group. The number of protons taken up after the formation of the protein-ligand complex changes with temperature (see Results section). Consequently, at about pH 7.1 and within the temperature range studied, a corresponding change in the ΔH_b with pH given by the following relation [29, 30] will take place:

$$\left(\frac{\partial \Delta H_b}{\partial \text{pH}}\right)_T = -2.3RT^2\left(\frac{\partial n}{\partial T}\right)_{\text{pH}} \quad (7)$$

The enthalpy change is negative and the entropy change is positive at the three studied temperatures. Thus, the binding of FdUMP to TS is favored by both enthalpy and entropy changes. The binding of nucleotide is noncooperative, which suggests that the interaction does not perform a conformational change affecting the other subunit. Therefore, its thermodynamic parameters may be attributed to intrinsic binding [31] and slight changes in the binding site region. This agrees with the observation that the crystal structure of TS bound to dUMP was nearly identical to the structure of free TS [4]. A low negative ΔC_p value, as we have obtained in this study, is usual for this type of processes [26]. The cooperative binding process and the induced structural change responsible for this cooperativity are usually accompanied by positive ΔC_p values [25].

The experimental values of enthalpy, entropy change (Table 1), and heat capacity change are the result of contributions with different sign: polar contributions arising from net formation of hydrogen bonds and electrostatic interactions; van der Waals' interactions between polar and apolar groups; contributions arising from hydration of ligand and protein groups; the ionization or protonation of groups of the ligand and/or protein.

When the protonation or ionization enthalpy is small compared with the binding enthalpy change, it is possible to obtain a reliable estimation of thermodynamic functions from structural information (Protein Data Bank) by Murphy's approach [32, 33]. The agreement between the values calculated from accessible surface areas and the calorimetric data should allow for an estimate of apolar and polar contributions to changes of thermodynamic parameters [34, 35].

During the binding of FdUMP to TS, different numbers of protons are taken up at 16, 25, and 35°C. Although the functional groups that are responsible for proton uptake have not been identified, the obtained results show clearly that the contri-

bution of protonation to ΔH_b is large enough to be ignored. Since Murphy's approach only gives us an estimation of the contribution of hydration processes, the results of its application to this system cannot be compared with experimental values unless the heat of the protonation of the FdUMP-TS complex is previously determined.

Crystallographic studies show several hydrogen bonding interactions between TS and dUMP [4], which may be taken as structural sources for the negative ΔH_b value, and in an analogous association, between FdUMP and the enzyme. Four highly conserved arginines (Arg23, Arg218, Arg178′, and Arg179′) and Ser219 are involved in an extensive hydrogen-bonding network around the phosphate [4–6, 28]. Some of these arginine residues, Arg23 and Arg218, have been shown to be important for activity [36]. Tyr261 and His259 are hydrogen bonded to the ribose ring and Asn229 is bonded to both O4 and N3 of dUMP. The net formation of hydrogen bonds produces a negative ΔH_b and should be accompanied by a negative ΔS. However, one should consider that the formation of hydrogen bonds between dUMP and the protein results in the release of water molecules. These molecules, before binding, were hydrogen bonded to protein and/or ligand; one could therefore expect unfavorable enthalpic and favorable entropic components due to the dissociation of water from protein or ligand or both [37].

Our results show that the binding of FdUMP to TS is both enthalpy driven and entropy driven in the range 16–35 °C and is accompanied by the uptake of protons. In addition, our data are in agreement with the observation that the binding of FdUMP does not induce a profound conformational change in the TS dimer, only certain slight modifications in the active site region. However, the affinity of this inhibitor for the enzyme is practically the same in the studied temperature range. The enthalpy change of binding is not strongly temperature dependent, arising from a small negative ΔC_p of binding which suggests only slight changes in the apolar surfaces accessible to the solvent.

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