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Relative free energies of binding to thymidylate synthase of 2- and/or 4-thio and/or 5-fluoro analogues of dUMP

Adam Jarmuła[a,*], Piotr Cieplak[b], Andrzej Leś[c] & Wojciech Rode[a]
[a]*Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., 02-093 Warszawa, Poland;* [b]*Accelrys Inc., 9685 Scranton Rd, San Diego, CA 92121, USA;* [c]*Chemistry Department, Warsaw University, 1 Pasteur St., 02-093 Warszawa, Poland*

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Summary

Free energy perturbation calculations have been applied to evaluate the relative free energies of binding of 2′-deoxyuridine-5′-monophosphate (dUMP) and its 2- and/or 4-thio and/or 5-fluoro analogues to the wild-type *E. coli* thymidylate synthase (ecTS). The results accurately reproduce experimentally measured differences in the free energy of binding of dUMP *versus* 5-fluoro-dUMP to thymidylate synthase. They indicate that preferred binding of dUMP compared to 5-fluoro-dUMP in the binary complex is equally related to (i) more favorable electrostatic interactions of the dUMP molecule in the enzyme active site, and (ii) its less favorable solvation in the aqueous solution. The relative free energies of binding in the binary complex show moderate and qualitatively indistinguishable discrimination among the studied fluorinated and non-fluorinated 2- and/or 4-thio analogues of dUMP. The binding free energies of monothio analogues of dUMP and 5-fluoro-dUMP correspond quite well with experimentally measured activities of these nucleotides in the thymidylate synthase reaction. On the other hand, the binding free energies of both dithio analogues, 2,4-dithio-dUMP and 2,4-dithio-FdUMP, show lack of such correlation. The latter suggests that very low activities of the dithio analogues of dUMP and 5-fluoro-dUMP may relate more to the covalent reaction of these nucleotides within the ternary complex with TS and 5,10-methylenetetrahydrofolate, than to their pre-covalent binding. We speculate that a lack of substrate activity of 2,4-dithio-dUMP is related to the high aromaticity of its pyrimidine ring that prevents the Michael addition of the active site cysteine thiol to the pyrimidine C6 atom. A stronger affinity of the fluorinated analogues of dUMP to thymidylate synthase, compared to the non-fluorinated congeners, results from the fluorine substituent producing a local strain in the C6 region in the pyrimidine ring, thus sensitizing C6 to the Michael addition of the cysteine thiol.

Abbreviations: CH_2THF – 5,10-methylenetetrahydrofolate; dCMP – 2′-deoxycytidine 5′-monophosphate; dTMP – 2′-deoxythymidine 5′-monophosphate; dUMP – 2′-deoxyuridine 5′-monophosphate; FdUMP – 5-fluoro-dUMP; S^2-dUMP – 2-thio-dUMP; S^4-dUMP – 4-thio-dUMP; $S^{2,4}$-dUMP – 2,4-dithio-dUMP; S^2-FdUMP – 2-thio-5-fluoro-dUMP; S^4-FdUMP – 4-thio-5-fluoro-dUMP; $S^{2,4}$-FdUMP – 2,4-dithio-5-fluoro-dUMP; TS – thymidylate synthase; FEP – Free Energy Perturbation; MD – molecular dynamics; K_D – dissociation constant; K_i – inhibition constant; K_m – Michaelis constant; ΔG_{sol} – free energy difference in aqueous solution; ΔG_{prot} – free energy difference in enzyme; $\Delta\Delta G_{bind}$ – relative binding free energy.

Introduction

Thymidylate synthase (TS) (EC 2.1.1.45), a target enzyme in chemotherapy, catalyzes the 5,10-methylenetetrahydrofolate-dependent reductive methylation of dUMP to dTMP [1, 2]. A dUMP analogue, 5-fluoro-dUMP (FdUMP), which exhibits a strong mechanism-based inhibition effect against thymidylate synthase, is an active form of 5-fluorouracil, an approved pro-drug used in chemother-

*To whom correspondence should be addressed. E-mail: a.jarmula@nencki.gov.pl

apy [3, 4]. Many cancers, however, are not sensitive to, or develop resistance to, 5-fluorouracil, which in addition is highly toxic. The search for less toxic and more specific inhibitors of TS continues, with the approaches focused on analogues of 5,10-methylenetetrahydrofolate (CH_2THF) [5–8], as well as dUMP analogues substituted at C5, C2 or C4 atoms of the pyrimidine ring [9–13]. Recent kinetic studies demonstrated that introducing two thio substituents at the positions 2 and 4 in the pyrimidine ring of dUMP and FdUMP causes substantially weaker, in relation to the parent compounds, activities of the dithio-substituted analogues in the thymidylate synthase reaction. Although 2,4-dithio-FdUMP and both monothio analogues of FdUMP (2-thio-FdUMP and 4-thio-FdUMP; Figure 1) caused a time-dependent inactivation of TS, proving to be slow-binding inhibitors of the enzyme [13–15], the 2,4-dithio analogue appeared to be as much as 10^3–10^4-fold less active than either of its monothio congeners [13]. Accordingly, the substrate analogue, 2,4-dithio-dUMP exhibited no substrate activity, and was characterized as a very weak competitive inhibitor [13], whereas both monothio analogues of dUMP (2-thio-dUMP and 4-thio-dUMP; Figure 1) are good substrates of TS (with only slightly higher K_m values compared to dUMP) [16, 17]. Structural studies that followed, showed that the 2,4-dithio substitution in dUMP distinctly increases an aromatic character of the pyrimidine ring, thus weakening the double character of the C5-C6 bond and probably rendering the C6 atom passive toward the nucleophilic addition of the TS active site cysteine [18]. The same structural studies suggested a possibility of steric hindrances in binding of 2,4-dithio-FdUMP to TS. That suggestion derived from the observation that the pyrimidine ring in the crystallographic structure of 2,4-dithio-5-fluoro-2′-deoxyuridine was less planar, and its 2- and 4-thio, and 5- fluoro substituents were much more deflected from the least-square ring plane, than in the structures of the parent 5-fluoro-2′-deoxyuridine or either of its monothio analogues.

In the present study we used the Free Energy Perturbation (FEP) method [19, 20] to determine the relative binding free energies of the 2- and/or 4-thio and/or 5-fluoro analogues of dUMP to the wild-type *E. coli* thymidylate synthase. The relative binding free energy from the dUMP → FdUMP perturbation has been calculated in order to compare it to the experimental free energies of binding calculated from the dissociation constants (K_D) of the TS-dUMP and TS-FdUMP binary complexes. Therefore, the dUMP → FdUMP perturbation has been used as the reference system for checking the force field parameters and conditions applied to our FEP procedure. Our simulations reproduce well the experimentally measured differences in the free energy of binding of dUMP *versus* FdUMP to thymidylate synthase and thus reinforce the credibility of the global model used in all perturbations.

Among the fluorinated and non-fluorinated thio analogues of dUMP, the relative binding free energies in the binary complex differ from each other, by qualitative assessment, inessentially. Those free energies correlate quite well with the experimental activities of the monothio analogues of dUMP and FdUMP in the reaction catalyzed by TS. On the other hand, there is no such correlation in the class of the dithio analogues. We speculate that the activities of the dithio analogues are largely reduced, in comparison to dUMP and FdUMP, upon initiation of the covalent reactions in the ternary complex with CH_2THF.

Methods

Free Energy Perturbation

The detailed description of the free energy calculations is to be found in the papers of Beveridge and Di Capua [19], Kollman [20], and Lamb and Jorgensen [21]. Here, we only briefly outline the basics of the Free Energy Perturbation method.

The free energy difference between two states, A and B, described by the Hamiltonians H_A and H_B, can be expressed as:

$$G_B - G_A = [-kT \ln < \exp -(H_B - H_A)/kT] >_A, \quad (1)$$

where $<>_A$ represents an ensemble average over state A, k is the Boltzmann constant and T is the temperature. In order to properly evaluate the average, it is crucial that states A and B are similar enough so that all significant contributions to the average come from regions of the potential energy hypersurface that are well sampled in both states. Since the A and B states are usually quite dissimilar, the free energy difference is calculated as a sum of small contributions between a series of nonphysical intermediate states (windows), that are usually uniformly and closely spaced using the coupling parameter λ:

$$\Delta G = \sum_{i=0}^{n} \Delta G(\lambda_{i+1} - \lambda_i) \quad (2)$$

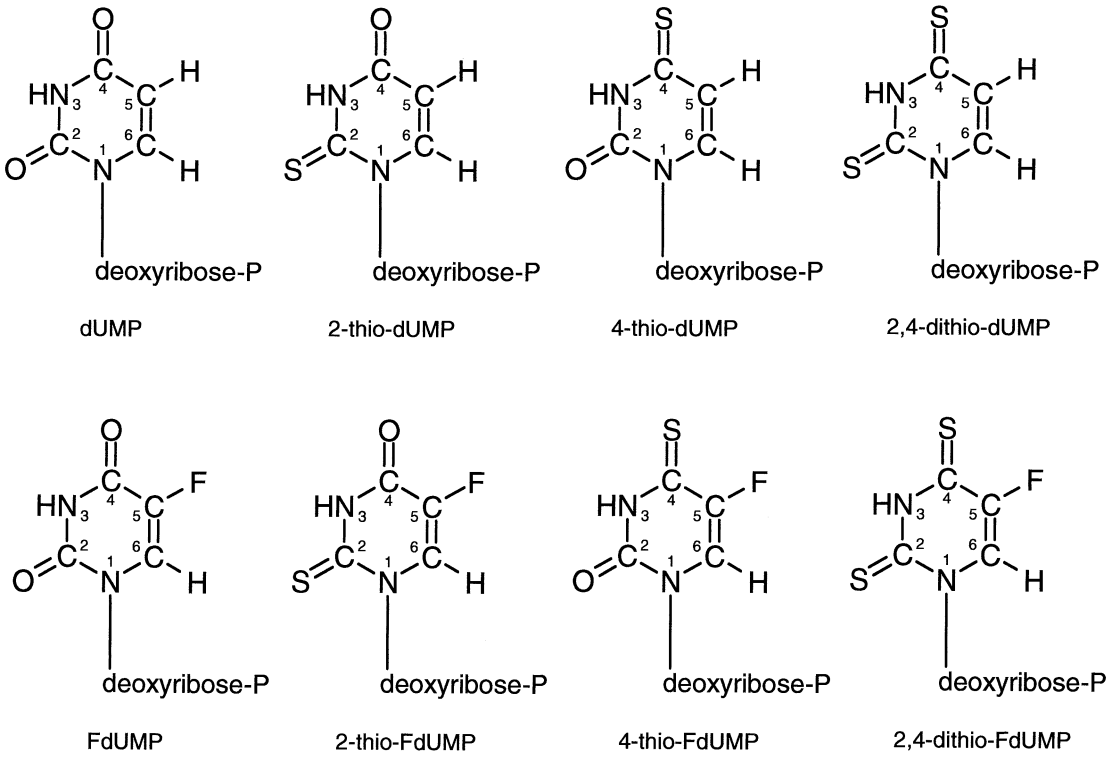


Figure 1. The analogues of dUMP and FdUMP included in FEP simulations.

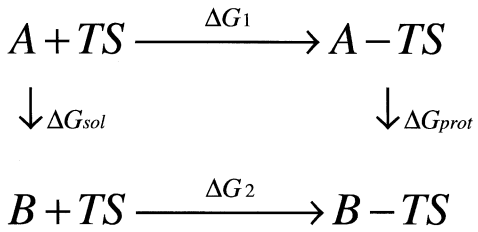
where

$$\Delta G(\lambda_{i+1} - \lambda_i) = -kT \ln < \exp[-(H_{\lambda i+1} - H_{\lambda i})/kT] >_{\lambda i} \quad (3)$$

$\lambda = 1$ can describe the initial state (A) and $\lambda = 0$, the final state (B). The method is called free energy perturbation since the force field parameters are coupled to the λ parameter and are perturbed from state A to B during molecular dynamics simulations by changing the λ parameter from its initial value of 1 to the final value of 0. For each intermediate value of λ the average value of $< \exp[-(H_{\lambda i+1} - H_{\lambda i})/kT] >_{\lambda i}$ is calculated over a chosen number of molecular dynamics steps.

The Free Energy Perturbation method was employed here to investigate the relative ligand-protein binding free energies using the thermodynamic cycle shown in Scheme 1. The A and B molecules refer to two different ligands (dUMP and its fluoro and/or thio analogue, respectively) perturbed into each other either in the aqueous solution (ΔG_{sol}) or in the active site of thymidylate synthase (ΔG_{prot}); and the relative free energies are determined from the following equation:

$$\Delta\Delta G_{bind} = \Delta G_{prot} - \Delta G_{sol} \quad (4)$$



Scheme 1. Thermodynamic cycle for calculating relative free energies of binding. ΔG_1 and ΔG_2 are absolute free energies of binding to enzyme of A and B, respectively. ΔG_1 and ΔG_2 could be obtained from experimental measurements but they are difficult to calculate. ΔG_{sol} and ΔG_{prot} represent nonphysical processes of the perturbations from A to B both in aqueous solution (ΔG_{sol}) and in the enzyme active site (ΔG_{prot}). Computationally, ΔG_{sol} and ΔG_{prot} can be calculated by means of the free energy perturbation method. As the thermodynamic cycle is closed and free energy is a state function, the total free energy change for the whole cycle, $\Delta G_{sol} + \Delta G_2 - \Delta G_{prot} - \Delta G_1$, is equal to 0, and hence the relative binding free energy is given by: $\Delta\Delta G_{bind} = \Delta G_2 - \Delta G_1 = \Delta G_{prot} - \Delta G_{sol}$.

Perturbations of dUMP to its 2- and/or 4-thio and/or 5-fluoro analogues

The relative free energies of binding to thymidylate synthase of the 2- and/or 4-thio and/or 5-fluoro ana-

logues of the dUMP molecule were investigated. Our simulations were initiated from the crystallographic complex of the highest available resolution. Among all binary non-mutant complexes between TS and dUMP that are stored in the Protein Data Bank, the *E. coli* TS-dUMP complex (at 2.2 Å; reference code 1bid [22]) has been chosen. The free energy perturbation calculations were performed on the supercomputer Cray-T3E using the AMBER 6.0 molecular modeling program [23], AMBER all-atom force field [24] parameters and default AMBER partial atomic charges for the amino acid residues. The partial atomic charges for the ligands have been derived from the electrostatic potentials calculated at the RHF/6-31G* theory level using the RESP method [25, 26]. The RESP charges for the atoms of the uracil part of the nucleotides are reported in Table 1.

In the case of the simulations in the TS active site, the crystallographic complex was solvated with a cubic box of TIP3P [27] water molecules extended up to 9 Å away from the solute. To ensure neutrality of the systems, the Na^+ and Cl^- counterions were added and the force field parameters were adopted from Aqvist [28] and Smith and Dang [29], respectively. During perturbation simulations, only residues within 13 Å distance from any atom of the ligand were permitted to move. We tested the relevance of the results obtained under such restriction by performing additional dUMP → S^2-dUMP perturbations with larger movable spheres of 14, 15 and 20 Å. Since the free energies obtained in all dUMP → S^2-dUMP perturbations showed very similar values (differing no more than 1.5%), the smallest tested movable sphere of 13 Å was used, in order to be able to complete the calculations in an efficient, less time-consuming way.

In the case of the unbound nucleotide simulations in the aqueous solution, the molecules were solvated with a cubic box of TIP3P water molecules extended up to 12 Å away from the solute. All atoms in the system were permitted to move. We realize that limiting the full motion area in the bound state, but not in solution, results in the boundary conditions being not equivalent in both related parts of the perturbation and that this might lead to the error cancellation being not fully invoked and cause an error increase. Nevertheless, the before mentioned size-dependency study of the ligand-protein complex showed nearly no dependence of the results on the size of the spherical active part. Besides, other important factors and parameters limiting the accuracy of the free energies such as equilibration procedure, cut-off radii, dielectric constants, force field parameters etc. were mostly exactly the same in both parts of the perturbation. This makes us believe that the simulations of the free ligand and ligand bound in TS active site were consistent to a sufficient extent to produce reliable results.

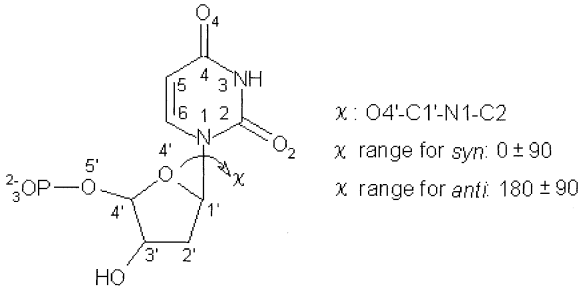


Figure 2. Torsion angle χ [53] describing the conformation around the glycosidic bond in dUMP (χ ranges are in degrees).

During perturbation simulations in the solution, we maintained a restraint on the torsion angle χ that determines the orientation of the nucleotide base in relation to its sugar (Figure 2). In the TS-dUMP binary complex crystal structure dUMP adopts a conformation with χ = *anti*. The dUMP molecule maintained invariantly the *anti* conformation during all simulations in the TS active site (minimization, molecular dynamics (MD) equilibration, and free energy perturbations). On the other hand, during the MD equilibration in the aqueous solution, dUMP spontaneously underwent conformational change such that χ was turning progressively to a high-*anti* and then *syn* conformation. The same *anti* to *syn* conformational change of dUMP during MD simulations in the solution has been observed before [30]. The authors related this conformational change to the large negative charge present on the phosphate. This charge induces the rotation of the base that results in the formation of a hydrophilic pocket consisting of O2, O4′, O5′, and PO_3^{2-}. Since the phosphate group is able to strongly polarize the surrounding water molecules, different orientations of the base with respect to the phosphate, observed in *anti* and *syn* conformations, result in different distributions of the water molecules. To test how this phenomenon would affect ΔG_{sol}, we performed two comparative dUMP → S^2-dUMP perturbations, one with unrestrained *syn* and the other with restrained *anti* conformation of the nucleotide. Inspection of the results revealed that solvation of the dUMP analogues strongly depends on the orientation of the effectively perturbed base with respect to the rest of the molecule (with a difference

Table 1. Partial charges for the uracil atoms in dUMP and its 2- and/or 4-thio and/or 5-fluoro analogues.

Atom	dUMP	S^2-dUMP	S^4-dUMP	$S^{2,4}$-dUMP	FdUMP	S^2-FdUMP	S^4-FdUMP	$S^{2,4}$-FdUMP
N1	−0.0612	0.1054	−0.0510	0.0798	−0.0793	0.0711	−0.0791	0.0340
C2	0.5080	−0.0815	0.4758	−0.0645	0.5223	−0.0626	0.5072	−0.0179
N3	−0.4144	−0.1275	−0.0804	0.1791	−0.4875	−0.1836	−0.1647	0.0336
C4	0.6203	0.6350	−0.0034	0.0268	0.6000	0.5926	−0.0403	0.0116
C5	−0.3613	−0.3776	−0.1000	−0.1299	0.1254	0.1036	0.3692	0.3158
C6	−0.1148	−0.0607	−0.1215	−0.0438	−0.2252	−0.1435	−0.2276	−0.1207
O2 / S2	−0.5473	−0.3647	−0.5376	−0.3567	−0.5398	−0.3555	−0.5354	−0.3505
O4 / S4	−0.5525	−0.5527	−0.3485	−0.3360	−0.5306	−0.5248	−0.2894	−0.2771
H3	0.3243	0.2433	0.2332	0.1428	0.3519	0.2639	0.2553	0.1917
H5 / F	0.1911	0.2019	0.1324	0.1369	−0.1736	−0.1590	−0.2225	−0.2075
H 6	0.1948	0.1660	0.1880	0.1525	0.2234	0.1846	0.2145	0.1740

in free energy between *syn* and *anti* conformations amounting to 25%). That result was not surprising, since similar dependence was observed formerly for the dUMP → dCMP perturbation in the aqueous solution [30]. Pyrimidine nucleotides and nucleosides are known to significantly prefer the *anti* conformation. Our former studies of the nucleoside analogues of all fluorinated nucleotides being discussed in this paper showed both their crystal and solution conformations being *anti* [18]. Therefore, the 10 kcal/mol torsion restraint has been applied to the χ angle of the perturbed nucleotides in order to maintain the *anti* conformation during simulations in the aqueous solution. This has been done also to secure an equivalency of the conformations used in both parts of the calculations: in the unbound and bound type simulations. The equilibrium value of χ of 151.7° has been adopted from the unrestrained minimization of dUMP in the aqueous solution.

All simulations were performed in the NpT ensemble with a constant temperature of 298 K and constant pressure of 1 atm, with periodic boundary conditions applied. The Berendsen coupling algorithm [31] and anisotropic diagonal position scaling were used to monitor the temperature and pressure, respectively. The temperature and pressure coupling constants were 0.1 and 0.4 ps, respectively. An atom-based cutoff of 10 Å was employed. The sampling of conformational space was performed with the use of molecular dynamics. At the initial steps of energy minimization, only water molecules were allowed to move, with the rest of the complex kept frozen. During the next steps of minimization, all enzyme residues and the ligands were not constrained and minimization was continued using the steepest descent and then conjugate gradient algorithms. Before initiating forward (λ: $1 \rightarrow 0$) perturbations, the minimized structures of the complexes were equilibrated at 298 K for 50 ps. The same equilibration procedure was repeated before backward (λ: $0 \rightarrow 1$) perturbations. Perturbations were performed using the Free Energy Perturbation Window Growth method. The entire ligand was used as the perturbed group and no intraperturbed group contributions to the free energy were calculated assuming that those contributions would cancel out, to some extent, between the TS active site and unbound type simulations. The bonds involving hydrogen atoms were constrained using the SHAKE [32] algorithm with a tolerance of 0.0005 Å. However, no bonds were restrained which were part of the perturbed group. Every perturbation run in the aqueous solution and in the TS active site has been divided into 401 and 251 windows, respectively. The single window widths were equal to $\Delta\lambda = 0.0025$ and 0.004, respectively. For each value of λ the 500 steps of equilibration/data collection were applied (eq/dc = 500/500) and the time step of 0.001 ps was used, which means that the entire single perturbation spanned a period of time of 401 ps in the solution and 251 ps in a more computationally demanding protein environment. This range of simulation time was long enough to warrant the convergence of our free energy results, since perturbations performed here involve very small changes in the molecular system, e.g., H atom into F and O atom into S.

Table 2. Free energy differences [kcal/mol] from perturbations in aqueous solution.

Perturbation	ΔG_{sol}		
	$\Delta G_{forward}$	$\Delta G_{backward}$	$\Delta G_{average}$
dUMP→S^2-dUMP	7.56	−7.49	7.52 ± 0.04
dUMP→S^4-dUMP	3.86	−3.85	3.86 ± 0.01
dUMP→$S^{2,4}$-dUMP	11.33	−11.32	11.33 ± 0.01
dUMP→FdUMP	−0.29	0.37	−0.33 ± 0.04
dUMP→S^2-FdUMP	7.18	−7.16	7.17 ± 0.01
dUMP→S^4-FdUMP	3.16	−3.14	3.15 ± 0.01
dUMP→$S^{2,4}$-FdUMP	10.27	−10.17	10.22 ± 0.05

Table 3. Free energy differences [kcal/mol] from perturbations in the thymidylate synthase active site.

Perturbation	ΔG_{prot}		
	$\Delta G_{forward}$	$\Delta G_{backward}$	$\Delta G_{average}$
dUMP→S^2-dUMP	7.60	−7.60	7.60 ± 0.00
dUMP→S^4-dUMP	1.18	−0.93	1.06 ± 0.13
dUMP→$S^{2,4}$-dUMP	10.47	−10.20	10.33 ± 0.14
dUMP→FdUMP	0.22	−0.41	0.31 ± 0.10
dUMP→S^2-FdUMP	6.78	−6.52	6.65 ± 0.13
dUMP→S^4-FdUMP	1.40	−1.48	1.44 ± 0.04
dUMP→$S^{2,4}$-FdUMP	7.30	−7.24	7.27 ± 0.03

Results and discussion

The results from our simulations are collected in Tables 2, 3 and 4. Table 2 presents the differences in free energy between dUMP and its 2- and/or 4-thio and/or 5-fluoro analogues in the aqueous solution (ΔG_{sol}), and Table 3 shows the free energy differences obtained from perturbations in the active site of thymidylate synthase (ΔG_{prot}). In Table 4 the differences between ΔG_{prot} and ΔG_{sol} ($\Delta G_{prot} - \Delta G_{sol}$) are collected, representing the relative binding free energies ($\Delta\Delta G_{bind}$) for all studied ligands bound non-covalently to TS. Estimated deviations of $\Delta\Delta G_{bind}$ in Table 4 represent an algebraic sum of the individual hystereses, i.e., the halves of the differences between forward and reverse ΔG_{sol} and ΔG_{prot} free energy changes. They are all, without exception, of low value. That the hystereses from forward and reverse perturbation runs in the aqueous solution (free state) are small (Table 2), may presumably be attributed, at least partly, to the fact that in the course of perturbations the ligands had their glycosidic bond restrained (see Methods and Figure 2). On the other hand, the unrestrained molecular dynamics in the TS active site (bound state) resulted in only slightly larger hystereses from forward/reverse perturbations (Table 3). Difficulties in accomplishing a meaningful and convergent sampling in the conformational space in complex molecular systems are well known obstacles that restrict some applications of the FEP method [33–35]. In our binary complex the ligand remains, to some extent, loose and can freely migrate during simulation from its crystallographic position in order to reduce unfavorable repulsive, and enhance favorable attractive, interactions. In view of that migration possibility alone, one could expect higher hystereses. However, our perturbed compounds differ by only one or a few substituents from each other and the simulations in the bound state were not of particularly long duration. Together, these circumstances were hardly expected to result in large conformational changes in the course of perturbations, and as such were conductive to a convergent sampling that resulted in the low hystereses for the bound state. However, since even in our relat-

Table 4. Relative binding free energies [kcal/mol] of the 2- and/or 4-thio and/or 5-fluoro analogues of dUMP.

Analogue	Perturbation	$\Delta\Delta G_{bind}$ calculated	$\Delta\Delta G_{bind}$ experimental
S^2-dUMP	dUMP→S^2-dUMP	0.08 ± 0.04	–
S^4-dUMP	dUMP→S^4-dUMP	-2.80 ± 0.14	–
$S^{2,4}$-dUMP	dUMP→$S^{2,4}$-dUMP	-1.00 ± 0.15	–
FdUMP	dUMP→FdUMP	0.64 ± 0.14	0.39;[38] 0.62[37]
S^2-FdUMP	dUMP→S^2-FdUMP	-0.52 ± 0.14	–
S^4-FdUMP	dUMP→S^4-FdUMP	-1.71 ± 0.05	–
$S^{2,4}$-FdUMP	dUMP→$S^{2,4}$-FdUMP	-2.95 ± 0.08	–

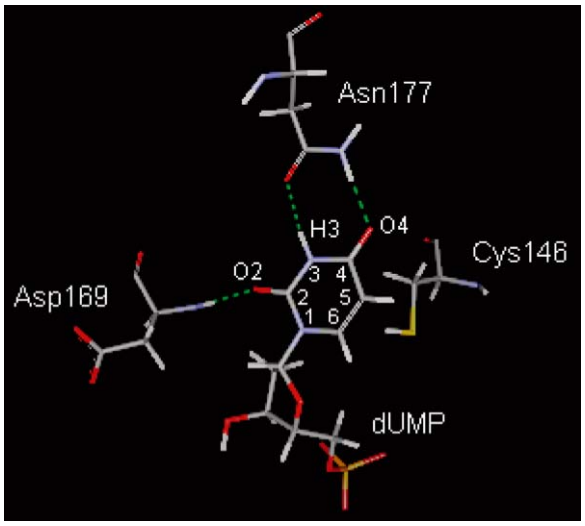


Figure 3. Hydrogen bonding interactions (dashed lines) between the uracil group of dUMP and the *E. coli* thymidylate synthase residues. Carbon atoms are colored in gray, oxygen atoms in red, nitrogen atoms in blue, sulfur atom in yellow, phosphorus atom in gold and hydrogen atoms in white. Cys146 in the binary complex is too distant from the pyrimidine ring of dUMP for the addition reaction at C6 to occur.

ively long perturbations, the conformational sampling might still not be sufficient, the estimation of $\Delta\Delta G_{bind}$ errors should be considered as a lower bound. We think that it should not affect the overall conclusions formulated in this paper, but the results should be treated as an estimation of trends rather than exact $\Delta\Delta G_{bind}$ values.

The initial structure of dUMP bound to TS (derived upon minimization of the respective crystallographic structure) was quite accurately regenerated after all reverse perturbations. This is very well reflected by the low values of the hystereses. In particular, the important hydrogen bonding network between dUMP O4 and N3-H3 and TS Asn177 (Figure 3), as well as other contacts, normally functioning as hydrogen bonds between dUMP and TS, including those in the phosphate-binding pocket [36], fell in the range of distances and in orientations permissible for hydrogen bonding.

The dUMP → FdUMP perturbation

The binding free energies obtained from the dUMP → FdUMP perturbation reproduce very well the experimental differences in binding free energy between both ligands (Table 4). The calculated free energy difference of 0.64 kcal/mol, nearly identical with the value derived from dissociation constants measured by Weichsel et al. [37], and comparable to the value emerging from dissociation constants published by Felder and co-workers [38], predicts slightly more preferred binding of dUMP than FdUMP to TS. Our results indicate that better stability of dUMP than FdUMP in the binary complex results from two, similar in value, contributions to the total free energy change of 0.64 kcal/mol: (i) interactions in the TS active site producing 0.31 kcal/mol, and (ii) interactions in the aqueous solution responsible for the remaining 0.33 kcal/mol. ΔG_{prot} for the dUMP → FdUMP perturbation is originating entirely from electrostatic interactions, whereas van der Waals interactions make almost zero kcal/mol energy contribution (results not shown). While ΔG_{prot} indicates that dUMP interacts more favorably with protein residues in the TS active site than FdUMP, the difference of 0.31 kcal/mol is quite small and seems difficult to rationalize. That is especially true for an open active site of mixed character, with both hydrophobic and polar residues, as well as many water molecules, which is the case for the TS active site in the binary complex (Figure 4). On the other hand, the aqueous solution is a medium of consistently strong polar character. Here, FdUMP

interacts slightly stronger with water molecules than dUMP because it has a higher dipole moment (the dipole moments calculated based on RESP charges are 33.39 D and 34.95 D for dUMP and FdUMP, respectively). In a consequence of combined effect, dUMP shows stronger binding to the enzyme, whereas FdUMP binds slightly weaker and relatively 'favors' the free state. It should be noted that in ternary complexes the binding rates for nucleotides are different, as upon binding of the folate cofactor or its analogue, leading to formation of a tight ternary complex, both dUMP and FdUMP have their binding rates usually considerably enhanced, but the enhancement is often greater for FdUMP, which is then stabilized better than dUMP [37, 39].

There is no evidence so far for an existence of structural differences in binding of dUMP and FdUMP to *E. coli* TS, but the fluorine atom substitution must implicate some changes in electron distribution in the uracil of dUMP. Those changes should affect a proton-acceptor ability of the O4 and O2 atoms, as well as a proton-donor ability of the N3-H3 moiety. Both O4 and N3-H3 make hydrogen contacts to the strictly conserved Asn177, and O2 is hydrogen-bonded to the side chain of Asp169 (Figure 3); thus even small fluctuations in the O2 and O4 proton-acceptor or the N3-H3 proton-donor abilities may alter the strength of those hydrogen bonds. While the RESP charges on the O2 and O4 atoms of dUMP and FdUMP (Table 1) differ very little and offer no explanation of this effect, a comparison of the pK_a values for N3-H3 dissociation in dUMP and FdUMP (9.3 and 7.8, respectively) [13] indicates that in dUMP the H3 atom is less acidic, which should result in slightly more stable hydrogen binding to the carboxylic δO of Asn177. It is not clear, however, how profoundly the energies associated with the aforementioned hydrogen bonds may contribute to the calculated free energy change in the bound state. In this context, it is worth noting that the mutagenesis studies showed that hydrogen bonds between Asn177 and dUMP do not contribute substantially to the dUMP binding energy [40, 41]. Instead, together with the shape of the binding cavity they help to achieve a proper orientation of the substrate inside the active site [42] and the latter contributes to catalysis [43].

In the binary complex the dUMP pyrimidine is located away from the active site cysteine (Cys146) thiol, such that the Michael addition to the pyrimidine C6 cannot occur and thus the reaction cannot readily begin in the absence of the cofactor (CH_2THF). Properly oriented dUMP forms a binding surface against which the pterin ring of the cofactor binds [44, 45]. The binding of the cofactor induces a conformational change of the enzyme, such that the C-terminal amino acids move largely and cover the active site [45–47]. In consequence, the reactants are sequestered from bulk solvent and the Cys146 is brought closer to the pyrimidine ring and into an orientation where thiolation at C6 can occur. At that time, a local reactivity at C6 may limit the rates of the thiol-adduct formation and thus cause a difference between dUMP and FdUMP binding to thymidylate synthase in the ternary covalent complex. The C6 region in FdUMP is potentially more reactive and so can be easier thiolated compared to dUMP. An electron-withdrawing inductive effect of the strongly electronegative fluorine in FdUMP acts toward lowering of the electron density on C6 and thus it sensitizes C6 to the nucleophilic addition of γS of Cys146. It should be noticed though, that an electron-releasing resonance effect of the fluorine compensates to some extent for the inductive effect as it sends some electron density back to C6 through conjugation with the C5 = C6 double bond. Theoretical elucidation of the fluorine influence on the local region in the ring is given by the Walsh-Bent rule [48–50]. According to it, a strongly electronegative ring substituent produces a local strain in the ring resulting in a local increase in reactivity (for more discussion of the effect of the fluorine on the pyrimidine ring of dUMP see Jarmuła et al. [51]).

Perturbations to the thio analogues of dUMP and FdUMP

The results presented in Table 4 point to a lack of distinct discrimination among the fluorinated and non-fluorinated 2- and/or 4-thio derivatives of dUMP, resulting from thermodynamic stability of their complexes with the enzyme. Inspection of the values of the two components of the binding free energy, ΔG_{sol} (Table 2) and ΔG_{prot} (Table 3), reveals their values to be considerably larger than the corresponding resultant $\Delta\Delta G_{bind}$ values. However, as they contribute to $\Delta\Delta G_{bind}$ with different signs, their entries tend to compensate for each other, resulting in a reduction of the $\Delta\Delta G_{bind}$ values. The contributions from ΔG_{sol} and ΔG_{prot} indicate that dUMP interacts more favorably, compared to its fluorinated and non-fluorinated thio analogues, both in the aqueous solution and within the TS active site. In approximation, that effect may originate from some similarity of both environ-

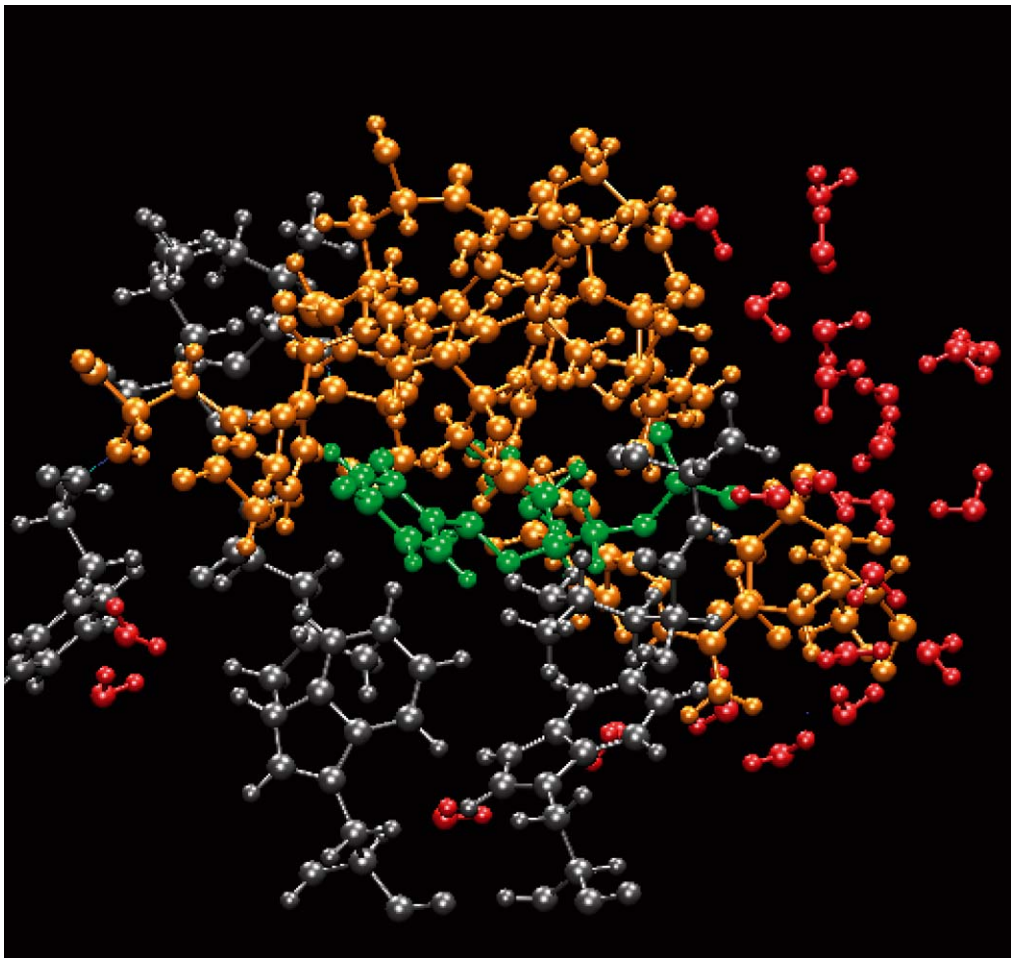


Figure 4. Ball and stick representation of the amino acid residues and water molecules in the proximity of dUMP in the solvated and minimized binary complex between *E. coli* TS and dUMP. Polar residues are shown in gold, hydrophobic residues in gray, water molecules in red and dUMP in green. This figure was prepared with the program VMD [54].

ments, as the active site in the binary complex is quite open and exposed to bulk solvent and at any given time during simulations many single water molecules and water domains reside in the active site. Nonetheless, the active site has no uniform character, as it is composed of the amino acid residues that are arranged in polar as well as hydrophobic domains (Figure 4). The aqueous solution, on the other hand, is a very strong polar medium that can effectively discriminate between dUMP and its more hydrophobic thio analogues. Therefore, the differences in G_{sol} contribute more substantially to $\Delta\Delta G_{bind}$ than differences in G_{prot} (cf. Table 2 *versus* Table 3). Since the ΔG_{sol} values are in favor of dUMP and disfavor its fluorinated and non-fluorinated thio analogues, the thio analogues relatively 'dislike' the water environment and tend to be expelled from it. In a combined effect from the perturbations in the unbound and bound state, the thio analogues show stronger binding to thymidylate synthase, whereas dUMP binds slightly weaker and relatively 'favors' the unbound state.

Exploring further the results in Table 4 we can sort the thio analogues of dUMP into two groups, depending on the level of correlation between the calculated binding free energies and the experimentally measured activities in the thymidylate synthase reaction. The first group consists of all fluorinated and non-fluorinated monothio analogues of dUMP. In the view of qualitative assessment, the compounds in this group are predicted to bind to thymidylate synthase with a similar strength, and, with one exception, slightly stronger than dUMP. In general, this corresponds quite

well with the substrate activities of S^2-dUMP and S^4-dUMP [16, 17], as well as the inhibitory potencies of S^2-FdUMP and S^4-FdUMP [14, 15] (represented by K_m and K_i, respectively), as measured for the reaction with L1210 and *L. casei* TS. Although dUMP shows a slightly lower K_m value (0.01 mM) in this reaction, both non-fluorinated monothio analogues are still good substrates of TS (0.02 mM and 0.07 mM, respectively), and the reaction of the fluorinated monothio analogues with TS renders the K_i values (0.07 μM and 0.6 μM, respectively) that are comparable to that of FdUMP (0.02 μM).

Tables 2 and 3 show considerable differences in the ΔG_{sol} and ΔG_{prot} values between the fluorinated and non-fluorinated 2-thio or 4-thio analogues of dUMP. While the 2-thio and 4-thio analogues ultimately produce qualitatively similar $\Delta\Delta G_{bind}$ values, S^2-dUMP and S^2-FdUMP have distinctly higher contributions from ΔG_{sol} and ΔG_{prot} than the corresponding S^4 analogues. This may suggest that the pyrimidine ring, both in the bound and free state, is less sensitive to the thio substitution at the C4 than at the C2 atom. However, the latter should be treated with caution, as analyzed separately the ΔG_{sol} and ΔG_{prot} free energies are of limited meaning because they are not corrected for internal terms.

In the second group, we can place both dithio analogues: $S^{2,4}$-dUMP and $S^{2,4}$-FdUMP. Here, the difference between the $\Delta\Delta G_{bind}$ values and the activities measured for thymidylate synthase reaction is very large. Although both $S^{2,4}$-dUMP and $S^{2,4}$-FdUMP are predicted to be stabilized in the binary complex with TS approximately as strongly as their respective monothio congeners and stronger (by 1.00 and 2.95 kcal/mol, respectively) than dUMP, their activities (31.5 μM and 26 μM, respectively) are much lower than those of the monothio congeners [13]. In addition, $S^{2,4}$-dUMP is the only representative among the non-fluorinated thio analogues of dUMP that exhibits no measurable substrate activity but behaves as a very weak inhibitor of TS.

It is characteristic for thymidylate synthase reaction that dUMP binding site and orientation become determined as early along the reaction path as upon formation of the binary complex. Despite some enhancement in binding, the orientation of the bound substrate remains virtually unaffected later in the reaction, when the ternary complex with the wild-type thymidylate synthase and the cofactor is formed [43]. In view of the latter, and considering our results, showing that the insertion of the two thio substituents at the positions 2 and 4 in the pyrimidine ring of dUMP or FdUMP preserves the strength of binding, we conclude that surprisingly low activities of $S^{2,4}$-dUMP and $S^{2,4}$-FdUMP may relate more to the covalent reaction than pre-covalent binding. As described in detail elsewhere [13,18], a high mobility of π-electrons from the carbon-sulfur bonds (C2 = S2 and C4 = S4; *cf.* Figure 1) leads to a highly aromatic character of the pyrimidine ring in 2,4-dithio-uridine. Since aromatic systems tend to preserve their electronic structures and so are inactive in addition reactions, the pyrimidine ring in 2,4-dithio-dUMP may be resistant to the thiolation at the C6 atom and this in consequence may decrease its activity. This explanation is plausible since it predicts that the thiol adduct at the pyrimidine C6 either does not form or forms at a very slow rate; otherwise it would be hardly understandable why $S^{2,4}$-dUMP, contrary to its monothio congeners, demonstrates the absence of substrate activity. It is also worth noting that the here proposed aromatic mechanism of action parallels to some extent the 'aromatic locking', a mode of TS inhibition caused by binding of the folate analogue, BW1843U89 [52]. The latter drug, bound strongly in the ternary complex via enhanced aromatic stacking with the pyrimidine ring of dUMP, prevented the Michael addition of dUMP to Cys146 by stabilizing the aromatic resonance form of dUMP over the saturated covalent complex at the C6 atom. On the other hand, with $S^{2,4}$-dUMP the lack of formation of the thiol adduct might also be a result of structural shifts or distortions in a local area in the active site. While we have found $S^{2,4}$-dUMP to bind strongly to thymidylate synthase in the binary complex, it is still not clear whether it is bound in the same manner as dUMP. If the dithio-substituted pyrimidine ring in $S^{2,4}$-dUMP shifts away from the position occupied in the active site by dUMP in order to bind more favorably with hydrophobic residues in the vicinity, this may result in a distorted binding of the cofactor and lead to a disfavored stabilization of the covalent complex at the C6 atom. Therefore, if the binding mode is considerably different than that of dUMP, it might prevent the thiolation at the C6 atom of the pyrimidine ring and thus prevent $S^{2,4}$-dUMP from exhibiting the substrate activity.

Comparing the inhibitory activities of the dithio analogues of FdUMP and dUMP, the fluorine-substituted analogue is a moderately stronger inhibitor, as reflected by the K_i values (26 μM and 31.5 μM, respectively) for the reaction with L1210 TS [13]. This gain in activity resembles the effect of

the fluorine substitution on the activity of dUMP. In both systems, FdUMP and $S^{2,4}$-FdUMP, the fluorine at C5 yields a local strain in the ipso region in relation to the substituent, resulting in the C5 = C6 double bond being more susceptible to the Michael addition of the thiolate anion of Cys146. In the case of the $S^{2,4}$-FdUMP system, the gain in activity with respect to $S^{2,4}$-dUMP occurs because the fluorine substituent, in addition to its above-mentioned role, also lessens the aromaticity and hydrophobicity of the pyrimidine ring.

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