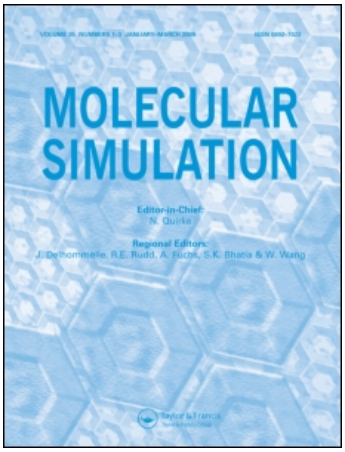

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Massimo Fusaro[a]; Agata Jurkiewicz[a]; Adam Jarmuła[b]; Andrzej Leś[a]; Wojciech Rode[b]
[a] Faculty of Chemistry, Warsaw University, Warsaw, Poland [b] Nencki Institute of Experimental Biology, Warsaw, Poland

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Hypothesis of a proton switch in QM/MM modelling of interaction of dUMP analogues with thymidylate synthase

Massimo Fusaro[a]*, Agata Jurkiewicz[a], Adam Jarmuła[b], Andrzej Leś[a] and Wojciech Rode[b]

[a]*Faculty of Chemistry, Warsaw University, Pasteura 1, Warsaw, Poland;* [b]*Nencki Institute of Experimental Biology, Pasteura 3, Warsaw, Poland*

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Quantum mechanical, molecular mechanics and molecular dynamics (MD) methods were used to investigate initial steps of 2′-deoxyuridine-5′-monophosphate (dUMP) methylation catalysed by the thymidylate synthase (TS) enzyme. The amino acid residues surrounding the active site within a 10 Å radius sphere were modelled with the combined quantum mechanical (B3LYP/LANL2DZ) and molecular mechanics ONIOM double-layer method. The results indicated the initial nucleophilic attack of Cys146 on dUMP to be concerted with formation of a hydrogen bond to the oxygen O4 of dUMP. Moreover, the proton in the vicinity of the O4 atom appears to act as a 'proton switch': if a proton is present near O4, it stabilises the S(Cys146)—C6(dUMP) sulphur–carbon bond, but if it is absent, the sulphur–carbon bond does not form. If the O4 oxygen is replaced by sulphur atom, the 'switch effect' does not occur. The suggested correlation between the strength of hydrogen bond involving O4 oxygen and the ability of dUMP to form bonds at C6 corresponds well to the crystal structures of TS complexes available in the Protein Data Bank. In the vast majority of crystal structures, the presence of the S(Cys146)—C6(dUMP) bond was coupled with the presence of hydrogen bond between the dUMP O4 atom and the conserved Asn177. The 'proton switch' hypothesis is supported also by the results of MD studies of TS binary complexes, suggesting that average distance separating S(Cys146) and C6(dUMP) becomes distinctly shorter in the presence of hydrogen bonding between Asn177 and O4.

Keywords: thymidylate synthase; dUMP; methylenetetrahydrofolate; density functional theory

1. Introduction

Thymidylate synthase (TS, EC 2.1.1.45) is an enzyme catalysing the C(5) methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate with concerted conversion of the cofactor, methylenetetrahydrofolate (MTF), to dihydrofolate [1]. This reaction is the sole pathway of *de novo* thymine synthesis in a cell and is a target in chemotherapy [2–5], in particular therapy involving fluoro-pyrimidine drugs [6,7]. Basic structure of the ternary complex TS–dUMP–MTF is presented in Figure 1.

The reaction of TS catalysis is initiated by the nucleophilic addition of the active site cysteine (CYS 146) to the pyrimidine C(6) atom of dUMP. It is believed that at this stage the negative charge of cysteine residue is delocalised, probably towards the C(4)═O of dUMP, forming the corresponding enolate anion at the C(4)—O(4) moiety. It is believed that due to resonance between C(4)═O(4), C(4)—C(5) and C(5)═C(6) bonds, the C(5) atom becomes negative enough to allow for a consecutive attack of the methylene residue which takes the form of an iminium cation created through the opening of imidazolidine ring of the cofactor [8].

A strong TS inhibitor, 5-fluoro-dUMP (FdUMP), is the active form of 5-fluorouracil and 5-fluoro-2′-deoxyuridine – drugs used in antitumour chemotherapy. The inhibition mechanism involves time-dependent formation of a ternary, covalently bound complex of the enzyme with FdUMP and N5,10-MTF. As the complex is formed, the reaction stops, resulting in a slowly reversible inactivation of the enzyme. The substitution of the C(4)═O group in FdUMP pyrimidine ring by either C(4)═N—OH group (in N4-hydroxy-5-fluoro-2′-deoxycytidine-5′-monophosphate; N4-OH-FdCMP) or C(4)═S group (in 4-thio-FdUMP) preserves high inhibitory potency, but alters selectivity of inhibition [9–11].

It is assumed that dUMP pyrimidine C(4)═O and non-dissociated N(3)—H groups are vital for the specific binding of pyrimidine moiety by the enzyme through active site asparagine residue (Asn177) and an ordered water molecule [12,13]. The assumption is that the asparagine residue stabilises, by hydrogen bonding, the partial negative charge on O4 of covalently bound dUMP [14]. This suggests an explanation of TS active site discrimination between dUMP and dCMP. As it is shown, strong mechanism-based inactivation by FdUMP or 4-thio-FdUMP of TS depends on the fact that N(3)—H group

*Corresponding author. Email: massimo@tiger.chem.uw.edu.pl

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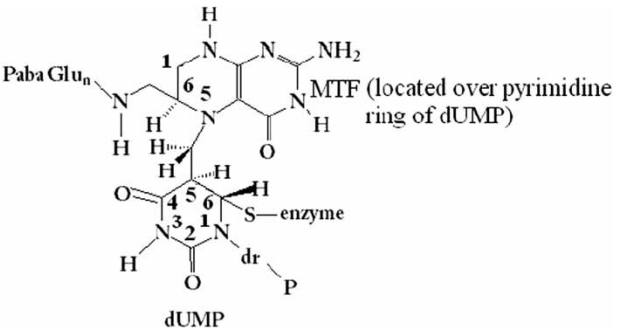

Figure 1. Schematic diagram of the TS–dUMP–MTF complex.

remains non-dissociated [15], hence it appears that the active site Asn177 is involved in the interaction. The same is probably true for N4—OH—FdCMP [8].

In order to learn more about the mechanism of interactions of dUMP and its 5-substituted analogues [FdUMP and 5-OH-dUMP (dUMP-OH)] with TS, and the role of 4-thio substitution in those interactions, the combined quantum mechanical and molecular mechanics modelling study was attempted and relevant data from molecular dynamics (MD) simulations of the structures of binary TS complexes with various other dUMP analogues, substituted at the C5 position in the base and/or in the C2′ position in the sugar, are presented. Findings from the computational work were compared with crystal structures of TS complexes available in the Protein Data Bank (PDB).

The numbering of residues of *Escherichia coli* TS is used in this work. The corresponding numbering of *Lactobacillus casei* TS is occasionally provided in parentheses.

The 'switch effect' hypothesis developed here can be summarised as follows: screening of the partial negative charge of O4 originating from the Cys146 attack on dUMP facilitates the formation of C6—S carbon–sulphur bond (so-to-say: no screening, no bond).

2. Method

2.1 *Models of the active site*

The active site of an enzyme contains the catalytic and binding sites. The structure and chemical properties of the active site allow for the recognition and binding of the substrate.

In other words, the active site is usually a small pocket at the surface of the enzyme, which contains residues responsible for the substrate specificity and catalytic residues that often act as proton donors or acceptors or are responsible for binding of a cofactor.

The X-ray diffraction structure of the monomer A of *E. coli* thymidylate synthase (ECTS) complexed with dUMP and MTF (PDB accession code 1KZI [16]) was used as the starting point for the calculations.

The hydrogen at C5 of dUMP was replaced either with fluorine, giving FdUMP, or with the hydroxyl group, giving dUMP-OH. The water molecules were removed from the X-ray structure.

Several models of different size that simulated the active site were considered. The small model, denoted as 5A model, was prepared by removing atoms farther than 5 Å from the S atom of CYS 146 residue, with the exception of atoms involved in non-peptidic bonds, which were included in the model. For the larger systems, denoted as 10A model, a similar procedure with the cut-off radius of 10 Å was adopted.

The residues included in our active site are consistent with the ones referred to in the literature [17]. In the 5A model of the active site, the following residues were included: TYR 94, LEU 143, ALA 144, PRO 145, CYS 146, HIS 147, ARG 166 and dUMP-X. The MTF cofactor was not included, as it is farther than 5 Å from the S atom of CYS 146. Moreover, to avoid unnatural expansion of the system, the border main chain atoms were frozen during the following optimisation at the experimental positions [18] (see Figure 2).

The 10A model contained the following residues: LEU 90, GLY 91, PRO 92, VAL 93, TYR 94, ALA 132, TRP 133, ASN 134,VAL 135, GLY 136, GLU 137, LEU 138, ASP 139, LYS 140, MET 141, ALA 142, LEU 143, ALA 144, PRO 145, CYS 146, HIS 147, ALA 148, PHE 149, LEU 163, TYR 164, GLN 165, ARG 166, SER 167, CYS 168, ASP 169, VAL 170, PHE 171, LEU 172, GLY 173,

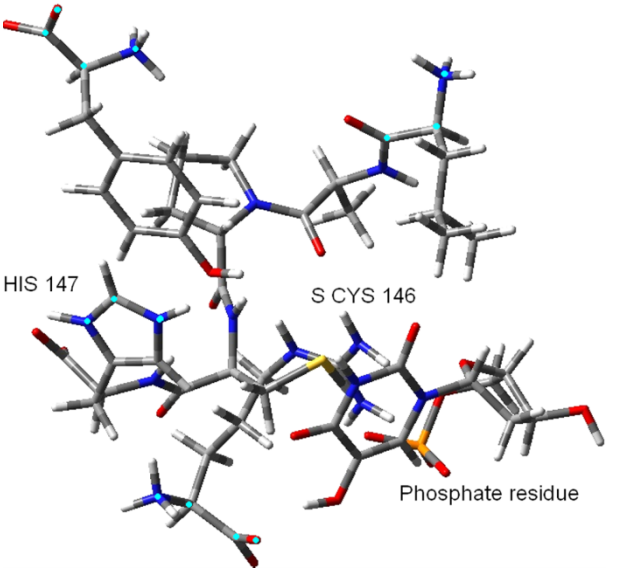

Figure 2. Schematic view of the 5A model. Fourteen atoms (C, N and O) indicated by cyan-coloured marks were frozen during the calculations to take the presence of the surrounding enzymatic environment into account (colour online).

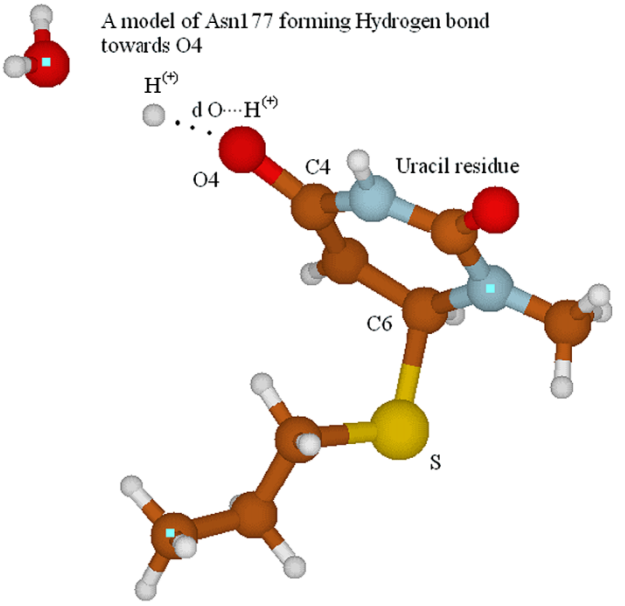

Figure 3. The S model of protonated residue approaching dUMP bond to CYS 146. The geometry corresponds to the transition state for proton movement. The atoms indicated by cyan-coloured marks, C, N and O of water, were frozen during the calculations.

LEU 174, PRO 175, PHE 176, ASN 177, ILE 178, ALA 179, SER 180, TYR 181, TRP 201, THR 202, GLY 203, GLY 204, ASP 205, THR 206, HIS 207, LEU 208, TYR 209, dUMP-X and MTF. In this case, also to avoid unnatural expansion of the system, the main chain atoms of the polypeptide were frozen in their experimental crystallographic positions, as in the work of Torrent et al. [18].

The dangling bonds in the models were saturated with hydrogen atoms.

The amino acid residues were modelled with the GaussView 3.07 [19] in their standard protonation states, adopting total charges of +1 (ARG, HIS and LYS), −1 (ASP and GLN) or 0 (all remaining amino acids).

The MTF cofactor had total charge of +1 and the phosphate residue had total charge of −2 ($—PO_4^{2-}$; 5A model) or −1 ($—HPO_4^-$; 10A model).

In the 10A and 5A models, a covalent bond exists between the S(Cys146) and C6 (dUMP) [16], and a proton is bonded to O4 atom of dUMP.

The resulting total charge of all models was neutral, since that allows for indirect simulation of the presence of counterions in the real systems. All models were prepared with the Chimera molecular visualisation program [20] and GaussView software [19].

In order to investigate the factors involved in the first step of the enzymatic reaction [17], i.e. the Michael addition of CYS 146 to the dUMP residue, a small S model was also created from a fragment of the 5A model, i.e. the coordinates of the atoms present in this small model are extracted from 1KZI PDB structure [16] (Figure 3). The S model simulates the approach of protonated residue (modelled as H_3O^+) towards the O4 oxygen of dUMP bound to Cys146 (modelled as 1-methyl uracil-6-mercaptopropane anion). The hydrogen atoms were added to saturate bonds and two carbon atoms were frozen during geometry optimisation to avoid unnatural expansion of the system.

2.2 *Calculations*

For the 5A and S models, the initial geometries based on literature data appropriately adapted were optimised with constraints using the quantum mechanical density functional theory with the B3LYP functional and the LANL2DZ [21] atomic basis set. The 5A models consist of 157 or 158 atoms (the latter in the case of dUMP-OH).

In order to incorporate protein environment effects to a larger extent, 10A models of the active site were also investigated. These models were subjected to the combined quantum mechanical/molecular mechanics (QM/MM) routine with mechanical embedding (two-layer ONIOM-ME) [22]. The internal part of the model (containing the same residues as in the 5A model) was investigated using the B3LYP/LANL2DZ method [18] (the QM-layer), whereas molecular mechanics method, employing the Amber force field [23], was applied for studying the external part (the MM-layer). In total, 149(150) and 737 atoms were included in the QM and MM layers, respectively.

All calculations were performed with the Gaussian 03 software [24]. The initial (before optimisation) and final (after optimisation) geometries were compared using the Tinker program [25].

3. Results

3.1 *Active site geometry*

The total QM/MM energies of the 5A and 10A models, as well as the mass-weighted, all-heavy-atom root mean square deviations (RMSD) between the initial and final geometries, are presented in Table 1.

Table 1 shows that the energy differences between the modelled systems depend almost entirely on the QM, while very little on the MM, parts; the former differing strongly from each other, while the latter agreeing very closely.

The RMSD between initial and final geometries of the 10A models are less affected during the optimisations than the corresponding RMSD in the 5A models due to more rigorous constraints imposed (freezing of main chain atoms in the 10A models vs. only border atoms in the 5A models).

Table 1. QM and MM energies and all-heavy-atom RMSD from the initial geometries in the 5A and 10A models.

	5A model		10A model	
System	Energy (Hartree) QM	RMSD (Å)	Energy (Hartree) QM/MM	RMSD (Å)
dUMP	−4048.1034	0.64	−3896.0053/91.9172	0.47
5-OH-dUMP	−4123.2206	0.86	−3971.2453/92.4893	0.48
FdUMP	−4147.3318	0.62	−3995.2899/92.0936	0.47

It was observed that during the geometry optimisation, when the proton connected to the oxygen O4 atom was removed (Figure 5), the distance separating the S (CYS 146) and C6 (dUMP-X) atoms lengthened considerably with respect to the initially assumed configuration (Figure 4). This result suggests that the formation of the S(Cys146)—C6(dUMP) bond is correlated with the presence of a hydrogen atom (or the formation of a strong hydrogen bond) at the O4 position of dUMP. Additional computational experiments showed that the S—C6 bond does not break if the O4 atom of dUMP is replaced with a sulphur atom or a thiol group. The S—C6 bond persists independently on the presence or absence of hydrogen bond at S4.

We can hypothesise that the hydrogen atom bound to the O4 atom of dUMP acts as a 'proton switch' stabilising the S(CYS 146)—C6 bond during the reaction. However, if the O4 atom is replaced with =S or —SH, the 'switch effect' is absent (presumably due to different charge distributions on the sulphur vs. oxygen atoms). The latter effect seems to contribute to the previously observed influence of the 4-thio substitution in FdUMP, consisting in a different specificity of slow-binding inhibition by 4-thio-FdUMP (of TSs from various sources) as compared to the specificity of inhibition by FdUMP [10].

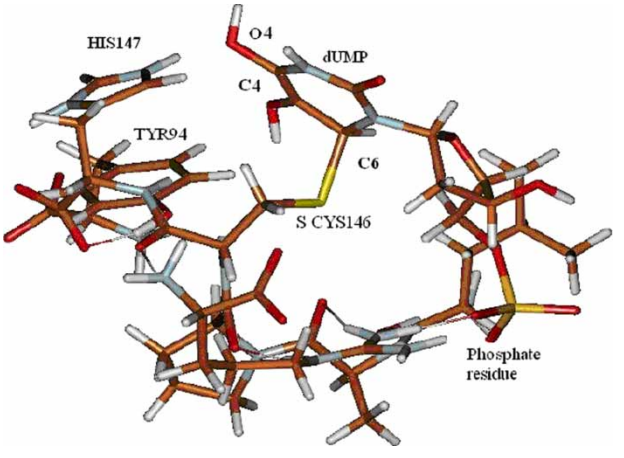

Figure 4. The final optimised structure of 5A model. The O4 atom of dUMP is protonated (zero total charge). The bond of CYS 146 S atom remains stable (1.8 Å as compared to 1.7 Å in 1KZI PDB file) during the geometry optimisation.

Additional calculations showed that the 'switch effect' occurs also in C(5)-substituted analogues of dUMP, i.e. when the hydrogen at the C5 position of dUMP is replaced by one of the following functional groups: —OH, —F, —OCH_3 or —CH_2OH. The ionisation state of the phosphate group (—PO_4^{2-}, —HPO_4^- or —H_2PO_4) and consequently the total charge of the system (neutral system with proton bonded to the O4 atom and the phosphate group doubly ionised) have not influenced the 'switch effect'.

A transition state quantum mechanical calculation was performed, using the small S model, to show whether the presence of a proton close to the oxygen atom O4 can stabilise the S(CYS 146)—C6 bond, see Figure 3. The transition structure with only one imaginary frequency corresponding to the proton transfer from O4 atom to the water molecule was found at the O–H distance of 2.36 Å. Our simplified model corresponding to the vacuum rather than to biochemical systems does not contain either solvent or enzyme environment, hence the overestimation of calculated energy barrier of around 80 kcal/mol. A real barrier, however, must be considerably smaller as the proton transfer is driven by electrostatic forces which are screened by the molecular residues of the environment.

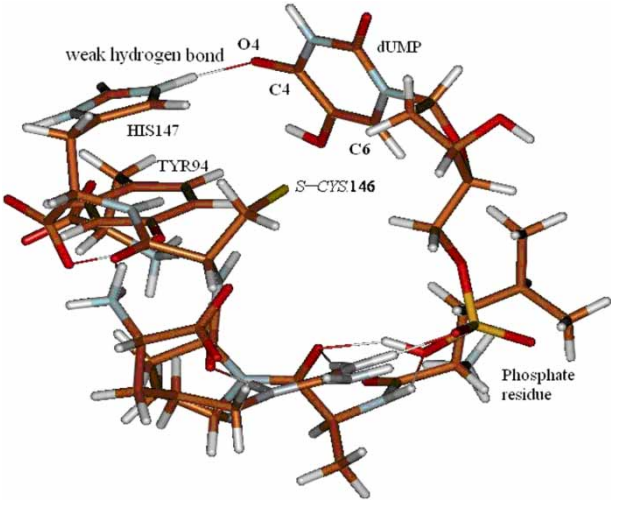

Figure 5. The final optimised structure of 5A model. The O4 atom of dUMP is not protonated. The bond of Cys146 S atom with C6 is not stable (>6 Å) during the geometry optimisation unless there is a proton connected to the O4 atom as shown in Figure 5. This is consistent with the reaction pathway described in [16]. The total charge of the model shown in the picture is zero.

Due to the presence of environment, the effective Coulombic forces are inversely proportional to the relative dielectric constant ε, which amounts to about 4 in the catalytic site interior in TS [4]. As a consequence, the forecast barrier should be reduced correspondingly. Moreover, experimental research shows [19] that the abstraction of the O4 proton in fact does take place. Therefore, the proton transfer can occur following the mechanism suggested here as the proton switch effect. The present simulation also indicates that the proton approaching dUMP residue bound to Cys146 can be an essential factor modulating the first step of the enzymatic reaction catalysed by TS.

3.2 Molecular dynamics

In the crystal structure of the ECTS–dUMP complex (PDB code 1BID) [26], the dUMP molecule is located away from Cys146 in such a way that the Michael addition to the pyrimidine C6 cannot occur. A distance of 3.16 Å observed in this structure can be perceived as a reference distance between the Cys146 S atom and the dUMP C6 atom in the absence of the cofactor. For the reaction to start, the cofactor binding must occur, upon which the active site closes and Cys146 is brought closer to dUMP, rendering the Michael addition feasible. An important factor that ensures a proper alignment of the cofactor at the time of binding is the orientation of the dUMP molecule, the correctness of which depends to a significant extent upon the presence of a double hydrogen bonding between the N3–H (donor) and O4 (acceptor) groups of dUMP and the active site asparagine (Asn177) [27–29].

Recently, MD simulations of the series of binary complexes between ECTS and 5- and/or 2′-substituted dUMP analogues were carried out in order to determine the effect of substitution on the conformation of the active site [30,31]. Considering our 'proton switch' hypothesis, particular attention should be paid to the variation of the C6–S distance in relation to the level of protonation of the O4 atom, the latter estimated in terms of preservation – or the lack thereof – of hydrogen bond between O4 and Asn177 as observed in the MD results.

3.3 Average structures from MD simulations

Average structures from the MD simulations can be divided into two groups depending on how much they conform to the active site organisation present in the crystal structure of the ECTS–dUMP complex. In general, a single C5-substitution in dUMP has a little (most of the substituents) to moderate ($-C_2H_5$ group) effect on the organisation of the active site [31], resulting in its strong preservation (first group). A similar effect takes place in the case of a double fluorine substitution, if one of the substituents lies at C5 such as in the 2′,5-diF-dUMP molecule [32]. The second group consists of the structures of three complexes, TS–5-C_3H_7-dUMP, TS–2′-F-*ara*-dUMP (fluorine in the 'up' configuration) and TS–2′,2″-diF-dUMP, wherein the substitution at dUMP C5 and/or C2′ results in the reorganisation of the active site [30,31] which leads to a considerable change in the positions with respect to each other of the catalytic CYS 146 and the pyrimidine ring of an analogue of dUMP, and in turn to an elongation of the molecular distance between S(CYS 146) and C6(dUMP). While the S–C6 distance in the structures from the first group fluctuates very little, ranging from 3.27 to 3.40 Å, it rapidly increases in the structures from the second group, from 3.75 Å in TS–2′-F-*ara*-dUMP to 4.36 Å in TS–2′,2″-diF-dUMP and to 5.65 Å in TS–5-C_3H_7-dUMP (Table 2). Apart from the S–C6 distance elongation, changes to the active site organisation manifest themselves in the TS–2′-F-*ara*-dUMP and TS–2′,2″-diF-dUMP structures in a displacement of the molecule of dUMP analogue from the position held by dUMP in the crystal structure of the TS–dUMP complex, which results in the uracil N3–H (TS–2′,2″-diF-dUMP) and O4 (TS–2′-F-*ara*-dUMP and TS–2′,2″-diF-dUMP) losing hydrogen bonding with Asn177 (Table 2). While in the TS–2′,2″-diF-dUMP structure, but not in TS–2′-F-*ara*-dUMP, the O4 atom gains a compensating hydrogen contact with the

Table 2. Distances separating C6(dUMP) and S(CYS 146) atoms and the presence (+) or absence (−) of hydrogen bonding in the average structures of binary complexes, as predicted by the results of MD simulations.

			Hydrogen contacts of Asn177 with	
Complexes from	Complex	C(6)–S(CYS 146) distance (Å)	N3—H	O4
First group	TS–5-X[a]-dUMP[b]	3.27–3.40	+	+
	TS–2′,5-diF-dUMP[c]	3.39	+	+
Second group	TS–2′-F-*ara*-dUMP[c]	3.75	+	−
	TS–2′,2″-diF-dUMP[c]	4.36	−	−
	TS–5-C_3H_7-dUMP[a]	5.65	+	+

[a] X = —F, —Cl, —Br, —I, —CHO, —CH_3, —C_2H_5, or CH_2OH. [b] Data from Ref. [26]. [c] Data from Ref. [27].

hydroxyl of Tyr94; dislocation of the molecule of 2′,2″-diF-dUMP prevents its C6 atom from maintaining an approachable distance to Cys146.

The structure of the TS–5-C_3H_7-dUMP complex has a special active site arrangement among the members of the second group [28]. In this structure, the large size of the propyl moiety resulted in the pyrimidine ring twisting and adopting the *syn* orientation relative to the sugar moiety (different from the usual *anti* conformation present in the rest of simulated structures as well as in the ECTS–dUMP crystal structure) in order to dispose the propyl between the indole rings of the two tryptophanes, Trp80 and Trp83, pushing them further away from each other as compared to their 'normal' positions. This rearrangement strongly dislocated the position of CYS 146, but did not disturb the hydrogen bonding of the uracil O4 and N3–H groups with Asn177 (Table 2).

An additional argument supporting the hypothesis of 'switch effect' on the C(4)═O residue comes from the analysis of the Asn177–O4 distance during MD simulation of the TS dimer complexed with dUMP/FdUMP and the cofactor. The results are presented in Figure 6 (TS with dUMP and cofactor) and Figure 7 (TS with FdUMP and cofactor). The variation of the Asn177–O4 distance in both systems clearly shows high flexibility of proton location in the vicinity of the O4 oxygen. Thus, it may be inferred that the proton can move from Asn177 towards dUMP/FdUMP and temporarily forms a hydrogen bond.

In general, the results of MD remain in line with the 'proton switch' hypothesis by showing the S(CYS 146)—C6(dUMP analogue) molecular distance to become longer and thus less approachable (in view of the chances for the formation of covalent bond) upon the lack of hydrogen bonding between the O4 atom of dUMP and Asn177 residue of ECTS, whereas shorter and more approachable upon the presence thereof. However, the results suggest that the hydrogen bond at O4 can be a necessary but not sufficient condition for a successful CYS 146 approach to the C6 atom. The active site organisation similar to the one observed in the crystal structure of the native TS–dUMP complex seems a prerequisite for the latter to take effect.

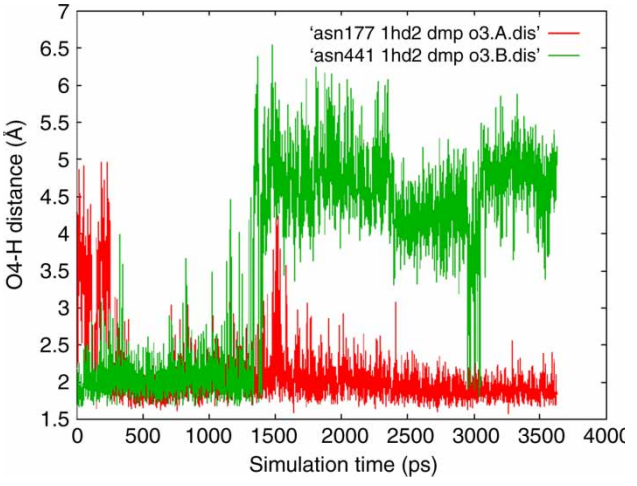

Figure 6. Distances (Å) between H atom of Asn177 (denoted as Asn441 in monomer B) and O4 atom of dUMP (denoted here as O3) for both monomers of TS, registered during 3 ns MD simulation of TS–dUMP–MTF ternary complex. Red, monomer A; green, monomer B (colour online).

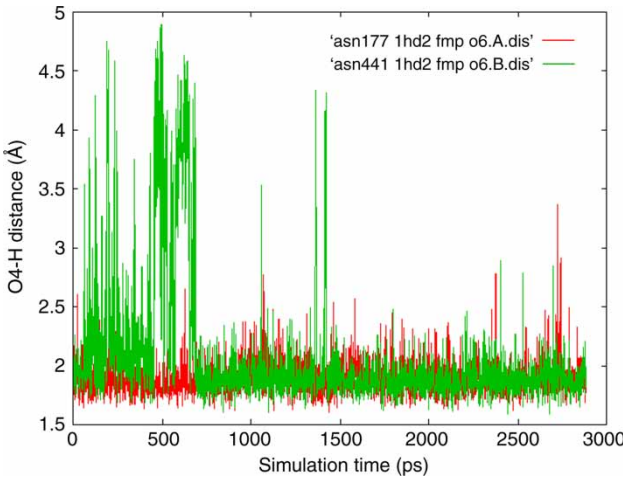

Figure 7. Distances (Å) between H atom of Asn177 (denoted as Asn441 in monomer B) and O4 atom of dUMP (denoted here as O6) for both monomers of TS, registered during 3 ns MD simulation of TS–FdUMP–MTF ternary complex. Red, monomer A; green, monomer B (colour online).

3.4 *Crystal structures from PDB*

Inspection of crystal structures of TS complexes stored in the PDB data bank shows that the presence of the S(Cys146)—C6(dUMP) covalent bond is correlated, in vast majority of cases, with the hydrogen bonding at the O4 atom. In most cases, one H-bond donated to O4 comes from Asn177, an essential amino acid residue aiding in recognising and orienting the molecule of dUMP. Additional hydrogen contact of O4 with Wat 1, a conserved water molecule participating in a water-mediated hydrogen bond network coordinated by glutamate 58, is usually also present. Only two structures were found, in which the H-bond between O4 and Asn177 is not present; the electron densities as well as distance and angular conditions, however, suggest the presence of the S—C6 bond. Both these structures are TS mutants: E58(60)Q from *L. casei* (LCTS) in complex with dUMP and a cofactor analogue CB3717 (PDB accession code 1VZE) [32], and N177A from *E. coli* (ECTS) in an analogous complex with dUMP and CB3717 (PDB accession code 1BQ1) [33]. In the structure of the LCTS–dUMP–CB3717 complex, dUMP shifted farther from its original position, with the O4 atom loosing the

hydrogen bonding with Asn177(229) and Wat 1, while forming a hydrogen bridge through another water molecule (Wat C) with Tyr94(146). In the structure of the ECTS–dUMP–CB3717 complex, on the other hand, asparagine 177 was mutated into a smaller residue of alanine, partly freeing a space occupied in the wild-type enzyme by the side chain of Asn177 and resulting in the side chain of His147 relocating into that space and forming a compensating hydrogen bond with O4. Another H-bond, linking O4 with the Wat C molecule, was also present.

Thus, in view of the crystal structures of TS complexes, hydrogen bonding to dUMP O4 in the form of direct bonding from the enzyme (Asn177) and/or an indirect bond, mediated by water, appears as a key factor for the presence of a covalent linkage between C6(dUMP) and S(CYS 146).

4. Conclusions

The nucleophilic attack of CYS 146 on the C6 carbon atom of dUMP appears to be concerted with the bonding of a proton to the oxygen O4 atom of dUMP or with the presence of a proton donor forming hydrogen bond with the O4 atom. Moreover, the proton bound to the O4 atom seems to act as a 'proton switch' which stabilises or destabilises the sulphur–carbon S(Cys146)—C6(dUMP) bond. The 'switch effect' does not occur when the O4 atom is replaced with a thione or thiol sulphur.

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The computational work was performed on the computers of the Interdisciplinary Centre for Mathematical and Computational Modelling, Warsaw University.

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