ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 1, pp. 21-28. © Pleiades Publishing, Ltd., 2007.
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Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-089, October 15, 2006.

Substrate Specificity of *Escherichia coli* Thymidine Phosphorylase

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Received April 4, 2006
Revision received August 16, 2006

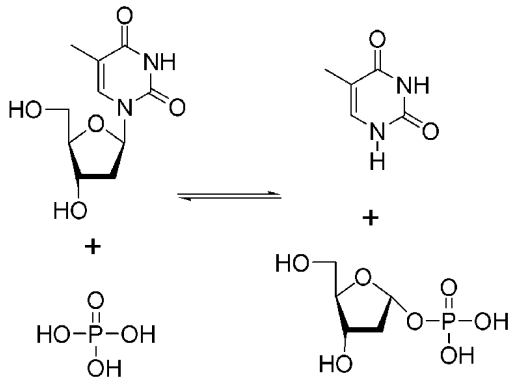
Abstract—Substrate specificity of *Escherichia coli* thymidine phosphorylase to thymidine derivatives modified at 5′-, 3′-, and 2′,3′- positions of the sugar moiety was studied. Equilibrium and kinetic constants (K_m, K_I, k_{cat}) of the phosphorolysis reaction have been determined for 20 thymidine analogs. The results are compared with X-ray and molecular dynamics data. The most important hydrogen bonds in the enzyme–substrate complex are revealed.

DOI: 10.1134/S0006297907010026

Key words: thymidine phosphorylase, thymidine derivatives, substrate specificity, inhibitors, analysis in terms of steric interactions

Nucleoside phosphorylases catalyzing nucleoside transformation reactions include thymidine phosphorylase (TP; EC 2.4.2.4), uridine phosphorylase (UP; EC 2.4.2.3), and purine nucleoside phosphorylase (PNP; EC 2.4.2.1). These enzymes are found in almost all organisms. Evolutionary discrepancies are small, and the homology between *Escherichia coli* and mammalian enzymes is >25% for UP, >33% for TP, and >20% for PNP [1]. The most specific enzyme is TP, whose substrate is thymidine. UP catalyzes phosphorolysis of both thymidine and uridine, whereas cytidine is not a substrate for either enzyme. The least specific is *E. coli* PNP, whose substrates are all purine nucleosides including both ribo- and 2′-deoxyribonucleosides. Relatively recently another enzyme, pyrimidine nucleoside phosphorylase (PyNP, EC 2.4.2.2.), was found in the thermophilic bacterium *Bacillus stearothermophilus*. This enzyme catalyzes phosphorolysis of thymidine and uridine, comprises two subunits with molecular weight of 46 kD each, and is homologous to TP of *E. coli* [1].

Nucleoside phosphorylases are involved in salvage pathways of nucleoside biosynthesis and catalyze a reversible cleavage reaction of a glycoside bond of a nucleoside with the release of free base and (deoxy)ribose-1-phosphate. Thus, TP catalyzes the following reaction [2]:



(1)

Nucleoside phosphorylases play a key role in nucleoside metabolism. Increased activities of these enzymes are observed in tumor cells; therefore, the enzymes can serve as markers for oncological diseases. The enzymes are used in industry for synthesis of drugs and commercially important nucleosides [3, 4]. By way of example, one can mention the known enzymatic conversion of nucleosides by UP and PNP in the presence of inorganic phosphate: β-D-arabinofuranosyluracil + adenine ↔ β-

Abbreviations: PNP) purine nucleoside phosphorylase; PyNP) pyrimidine nucleoside phosphorylase; TP) thymidine phosphorylase; UP) uridine phosphorylase.

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D-arabinofuranosyladenine + uracil. Moreover, the enzymes are widely used for quantitative determination of inorganic phosphate [5]. These facts stimulate great interest in these enzymes.

The aim of this work was to study substrate and inhibitory properties of thymidine derivatives in the reactions catalyzed by *E. coli* TP.

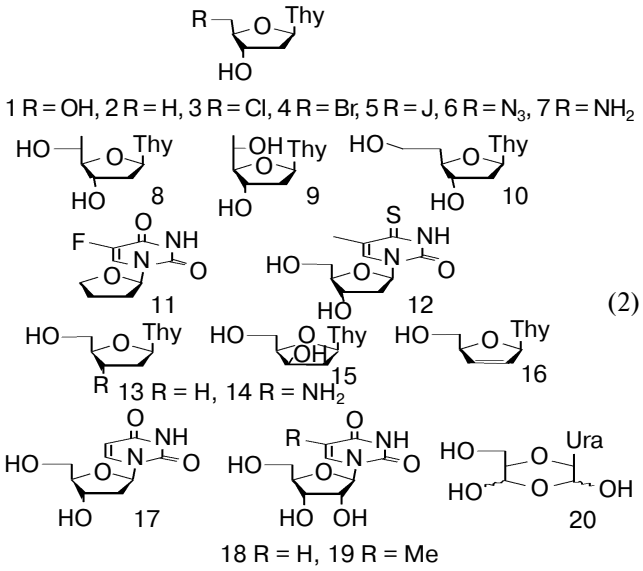
Though the main goal of nucleoside phosphorylase studies was and still is the search for efficient inhibitors [6-13], many investigations focus on substrate specificity of TP. However, it should be noted that kinetic constants have been measured predominantly for natural nucleosides and 5-substituted analogs of 2′-deoxyuridine [14-18], and the studied properties of modified oligonucleotides were limited to the determination of relative enzyme activity [19-21]. During the study of substrate specificity of TP from *E. coli* [19] and TP from liver cancer cells [20], it was found that the enzyme is sensitive only to 2′-deoxynucleosides. The relative rate of uridine phosphorolysis by TP from adenocarcinoma cells was 100 times lower than that of thymidine and 2′-deoxyuridine. 3′-Deoxyuridine and 5′-deoxyuridine were not substrates for this enzyme [20]. Thymidine derivatives, in which the 3′-hydroxyl group was replaced with azido and fluoro groups, were not substrates for *E. coli* TP either [19]. It was evident that the presence or absence of hydroxyl groups in the sugar moiety was necessary for the reaction to proceed.

Recombinant *E. coli* TP [22] was used in this work. Kinetic and equilibrium constants for enzymatic phosphorolysis were measured for thymidine derivatives with modified sugar moiety. The results are compared with X-ray and molecular dynamics data.

MATERIALS AND METHODS

The following reagents were used in this work: thymidine, uridine, 5-methyluridine, 2′-deoxyuridine (Sigma, USA); KH_2PO_4, CH_3COONa (Merck, Germany). Kinetic constants were measured using a Cary 300 spectrophotometer (Varian, Australia). High performance liquid chromatography was performed on a Gilson chromatographer (USA) using a column of 4.6 × 150 mm (5 μm, Nucleosil C-18). Structural analysis was carried out using the computer programs Protein Viewer and TURBO-FRODO [23].

The following nucleosides were obtained according to previously published protocols: 5′-deoxythymidine (**2**) [24], 5′-chloro-5′-deoxythymidine (**3**) [25], 5′-bromo-5′-deoxythymidine (**4**) [26], 5′-iodo-5′-deoxythymidine (**5**) [26], 5′-azido-5′-deoxythymidine (**6**) [27], 5′-amino-5′-deoxythymidine (**7**) [27], 1-(2′,6′-dideoxy-β-D-*ribo*-hexafuranosyl)thymine (**8**) [28], 1-(2′,6′-dideoxy-α-L-*lyxo*-hexafuranosyl)thymine (**9**) [28], 1-(2′,6′-dideoxy-β-D-*ribo*-hexafuranosyl)thymine (**10**) [28], 4-thiothymidine (**12**) [29], ftorafur (tegafur) (**11**) [30], 3′-deoxythymidine (**13**) [31], 3′-deoxy-2′,3′-didehydrothymidine (**16**) [31], 3′-amino-3′-deoxythymidine (**14**) [32], 1-(2′-deoxy-β-D-threo-pentafuranosyl)thymine (**15**) [33]. In solution, oxidized uridine derivative (**20**) [34] exists predominantly as a diastereomeric mixture of 1,4-dioxane derivatives. (Chemical structures of modified nucleosides are shown below.)



(2)

Determination of enzymatic activity of *E. coli* thymidine phosphorylase. Enzymatic activity of thymidine phosphorylase was determined spectrophotometrically at 25°C by continuously measuring the difference in absorption of a nucleotide (a product of enzymatic conversion of a heterocyclic base) at 290 nm. The calculations were based on $\Delta\varepsilon_{290}$ = 850 $M^{-1}\cdot cm^{-1}$ for the thymidine/thymine pair [29]. The reaction mixture contained (per ml): 0.5 μmol thymidine, 50 mM potassium phosphate buffer (pH 6.5), 0.18 TP activity units. For determination of initial rate of the reaction, the linear range of the kinetic curve (dependence of absorption on time) was used. One activity unit was defined as the enzyme amount that catalyzes the conversion of 1 μmol thymidine in 1 min at 25°C.

Protein amount was quantified using Lowry's method and by spectrophotometry. The protein content was calculated by Calcar's method based on absorption at 260 and 280 nm. TP concentration was 7.8 mg/ml, 1 activity unit was $0.72\cdot10^{-10}$ mol.

Determination of Michaelis constant for phosphorolysis of thymidine analogs. The Michaelis constant for phosphorolysis of thymidine analogs in the presence of TP was determined spectrophotometrically at 25°C by changing the substrate concentration in the reaction mixture from 100 to 1000 μM with fixed concentration of phosphate (50 mM, pH 6.5); total volume of the reaction mixture was 1 ml. The reaction was initiated by the addi-

tion of 0.03 unit of enzyme (0.3-0.5 unit for slow substrates), and the initial reaction rate was measured during 5 min on a Cary-300 spectrophotometer using kinetic mode at 290 nm.

The K_m value was determined using Lineweaver–Burk coordinates; Δε value for thymine/thymidine pair used for calculation of a reaction rate was 850 $M^{-1}\cdot cm^{-1}$ (at 290 nm). In the case of thymidine derivatives, the Δε value was taken equal to Δε for thymine/thymidine pair (850 $M^{-1}\cdot cm^{-1}$).

Determination of inhibition constants. Inhibition constants for compounds **3**, **4**, **5**, **6**, **12**, **13**, **14**, and **15** were determined spectrophotometrically [29]. The reaction was started by the addition of 0.03 enzyme units to the solution of substrate **12** (200 μM) in potassium phosphate buffer, and the initial rate of enzymatic phosphorolysis was measured at 355 nm in the presence of inhibitor. The inhibitor concentration was changed from 0 (control) to 1000 μM. Analogous measurements were carried out for substrate concentration of 400 μM. The K_I value was determined by Dixon's method using the double reciprocal plot of initial reaction rate vs. the inhibitor concentration for two different substrate concentrations.

Determination of equilibrium constants for phosphorolysis of thymidine and its analogs. Nucleoside + P_i ↔ base + dRib-P_i. The equilibrium constant was determined at different pH values (6.5, 7.5, 8.2) and temperatures (37 and 4°C). Given below is the description of the experiment at pH 6.5 and temperature of 37°C; for the other pH and temperature values the reagent amount and buffer concentration were not changed. To a solution of 0.2 μmol of thymidine derivative in 1 ml of Tris-HCl buffer (50 mM, pH 6.5), 2 μmol of potassium phosphate was added and the reaction initiated by the addition of 0.06-0.3 activity unit of *E. coli* TP. The amount of added enzyme was dependent on the reaction rate: more enzyme was added in the case of slow substrates. The reaction mixture was incubated at 37°C until equilibrium state was reached (1-2 h). The mixture was analyzed by high performance liquid chromatography on a column of 4.6 × 150 mm (5 μm, Nucleosil C-18) in a linear acetonitrile gradient from 0 to 15% during 15 min in 50 mM sodium acetate (pH 4.3). The signals were detected at wavelength 267 nm. The equilibrium constant was calculated according to the following formula:

$$K_{eq} = [\text{base}]\cdot[\text{dRib-1-P}]/[\text{nucleoside}]\cdot[P_i].$$

Equilibrium concentrations were determined considering the integral signals of heterocyclic base and nucleoside and their molar extinction coefficients, assuming that [base] = [dRib-1-P].

Determination of dependence of Michaelis constants and phosphorolysis reaction rates for thymidine (1) and 3′-amino-3′-deoxythymidine (14) on the pH of the buffer. The pH dependence of the Michaelis constant and phosphorolysis reaction rate for thymidine (**1**) and 3′-amino-3′-deoxythymidine (**14**) in the presence of TP was determined spectrophotometrically. For the measurement of K_m and reaction rate, a set of standard solutions of 50 mM potassium phosphate with pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 was used.

RESULTS

Effect of pH on K_m. The pH dependence of Michaelis constant and reaction rate of *E. coli* TP-catalyzed phosphorolysis for thymidine (**1**) was determined. The plot of dependence of pK_m on the pH upon TP-catalyzed reaction of thymidine is a bell-shaped curve with a narrow maximum, which is located at the pH value of 6.5 and bending at pH 6.1 and 6.8 (Fig. 1). The appearance of bending is usually associated with the involvement of an ionized amino acid group of the active site in the bond formation. The pK_m curve gives a pK value for a histidine imidazole group of 5.6-7.0 [35]. These data are in agreement with molecular dynamics calculations [36, 37] and suggest that His85 may be directly involved in binding.

Effect of pH on equilibrium rate of enzymatic phosphorolysis. The reaction mixture was incubated at pH 6.5 or 8.2 and at two different temperatures: 37 and 4°C. After the incubation of the reaction mixture at pH 6.5 (37°C) there was no distinct plateau corresponding to equilibrium (Fig. 2, curve *3*), whereas deoxyribose was chromatographically detected among the products. Based on the literature on instability of deoxyribose phosphate in acidic and neutral media [38], it was concluded that the shift in equilibrium is due to the chemical hydrolysis of one of the products. Since it is known that the rate of non-enzymatic hydrolysis is dramatically dependent on the temperature, we tried to exclude the influence of

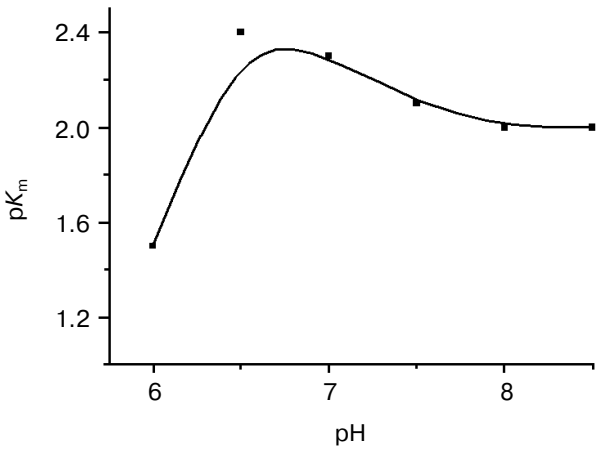


Fig. 1. Dependence of pK_m on pH for TP-catalyzed reaction of thymidine.

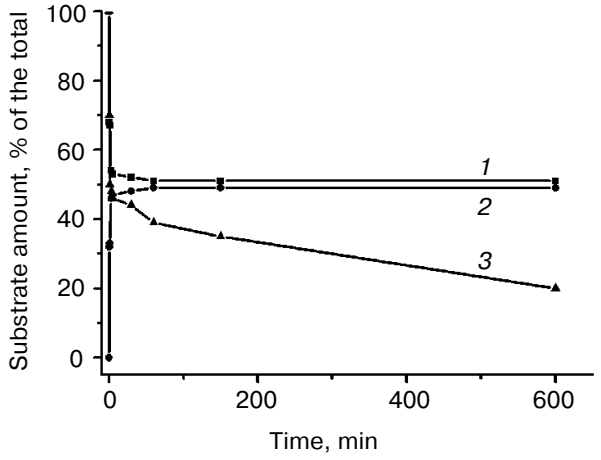


Fig. 2. Time dependence of the amount of substrate and product for the reaction of thymidine phosphorolysis. The K_{eq} values were measured in 50 mM Tris-buffer, nucleoside to phosphate ratio was 1 : 10. Curves: *1*) accumulation of thymine, pH 6.5, temperature 4°C; *2*) phosphorolysis of thymidine at pH 6.5 and 4°C; *3*) phosphorolysis of thymidine at pH 6.5 and 37°C.

hydrolysis and determined the equilibrium constant for thymidine phosphorolysis reaction at pH 8.2 and temperature of 4°C. The dependence of substrate amount on time shows a distinct equilibrium plateau, where the concentrations of base and nucleoside were constant during several hours (Fig. 2, curves *1* and *2*). The analogous experiments were performed using thymidine derivatives as substrates, and in all cases we observed equilibrium plateau only at pH 8.2 and 4°C.

Determination of kinetic constants of phosphorolysis reaction of modified nucleosides. Analysis of the literature shows that the main kinetic measurements (K_m, K_I, relative rate) were carried out for thymidine and its derivatives containing modified heterocyclic base [6, 19-21]. Using X-ray analysis followed by the molecular dynamics simulation, the structures of *E. coli* TP–substrate complex were obtained, and the main amino acids involved in binding of heterocyclic base [39] were determined. As seen from Table 1, the K_m value for thymidine (**1**) in the reaction catalyzed by recombinant TP is not different from the data obtained for an enzyme isolated from cells [16], and 2′-deoxyuridine (**17**) is comparable with thymidine (**1**) by its substrate properties.

Modification at 5′-position (derivatives 2-10). Data on the activity of 5′-deoxythymidine (**2**) are of interest. It not only binds well with the enzyme (K_m = 400 μM), but also is phosphorolyzed with the same rate as for thymidine (**1**).

The replacement of a hydroxyl group at 5′-position with a large substituent does not hinder nucleoside binding to TP. However, the reaction rate is significantly decreased with the increase in substituent dimension, as in the case of the 5′-chloro-derivative (**3**). Increase in the van der Waals radius of the substituent (compounds **4** and **5**) results in nucleoside binding to enzyme without phosphorolysis, whereas the 5′-azido-derivative (**6**) practically does not bind with the enzyme. The replacement of the hydroxyl group with the amino group (**7**) allows substrate binding with the same constant as for thymidine (**1**), but in the case of 5′-chloro-5′-deoxythymidine (**3**) the reaction rate decreases 100 times.

The analogous effect is observed when additional methyl group or methylene link are introduced at the 5′-position of thymidine (compounds **8-10**): the reaction rate decreases 100 times, but the binding constant remains the same. It is evident that hydroxyl group at 5′-position (and, probably, the hydrogen bond formed by this group) is not crucial for binding or for reaction.

Modification at 3′-position (derivatives 13-16). The absence of 3′-hydroxyl group or inverted configuration at C3′-atom: compounds **13-15** are not TP substrates ($k_{cat} < 10^{-4}$ sec^{-1}), though they inhibit the enzyme activity with K_I similar to K_I for thymidine (K_I = 400-600 μM).

3′-Amino-3′-deoxythymidine (**14**) is not a TP substrate at pH 6.5, but it undergoes phosphorolysis when the pH reaches 8.0. This can be associated with the presence of the 3′-amino group in the vicinity of the α-amino group of an amino acid residue in the active site; thus the two protonated groups are repelled. At pH above 8.0, the positive charges on nucleoside and protein amino groups disappear (pK of 3′-amino group is 8.26, pK of α-amino group is 7.6-8.4; the values are calculated using ACD/Labs Online (I-Lab) software). The mutual repulsion disappears and a hydrogen bond necessary for the reaction can be formed.

2′,3′-Didehydro-2′,3′-dideoxythymidine (**16**) is an enzyme substrate characterized by the same Michaelis constant as thymidine (K_m = 350 μM), but the reaction rate decreases more than 100 times and equilibrium is not reached. It is well known that 2′,3′-deoxynucleosides are unstable and can be easily hydrolyzed under acidic pH (pH < 7.0) [40, 41]. The rate of acidic hydrolysis for 2′,3′-didehydro-2′,3′-dideoxy-derivatives of purine and pyrimidine nucleosides is 2000-2500 times higher than for ribonucleosides, and 500-1000 times higher than for 2′-deoxynucleosides. It is obvious that the rate of 2′,3′-didehydro-2′,3′-dideoxyribose phosphate is much higher than the rate of the enzymatic phosphorylation reaction; therefore, the equilibrium is shifted and the constant can not be determined. The possibility of nonspecific hydrolysis was controlled by incubation without the enzyme.

The absence of 3′-hydroxyl group is not important for binding of nucleoside to the active site, but dramatically influences the reaction rate. It is probable that the hydrogen bond formed by this group fixes furanose residues in a conformation that is necessary for nucleophilic attack by phosphate (in-line mechanism).

Modification by 2′-position: uridine derivatives 18-20. Uridine and methyluridine (**18** and **19**) are not TP

Table 1. Kinetic parameters of enzymatic phosphorolysis of thymidine and its derivatives measured spectrophotometrically (50 mM potassium phosphate buffer, pH 6.5, 25°C; the K_{eq} values were determined in 50 mM Tris-buffer, pH 8.2, 37°C, nucleoside/phosphate ratio was 1 : 10)

Nucleoside	*E. coli* TP			
	K_{eq} ($1/K_{eq}$)	K_m (K_I), μM*	k_{cat}, sec^{-1}**	k_{cat}/K_m
1	0.07 (14.3)	300	198.0	0.66
1 [2, 25]	0.10	380		
PO_4^{3-} [25]		890		
2	0.05 (20.0)	400	260.0	0.64
3	0.07 (14.3)	300	1.7	0.006
4	—	(400)	—	—
5	—	(400)	—	—
6	>0.01	—	—	—
7		400	0.58	0.0015
8	0.14 (7.0)	330	0.1	0.0003
9	0.10 (10.0)	350	0.45	0.0013
10	0.068 (14.6)	300	0.6	0.002
11	—	(450)	—	—
12	0.07 (14.3)	280	63.0	0.25
13	—	(850)	—	—
14	—	(600)	—	—
14 (pH 8.0)		400	1.1	0.0027
15	—	(450)	—	—
16	—	350	0.4	0.0011
17	0.04 (25.0)	320	35.0	0.18
18	—	(60)	—	—
19	—	(100)	—	—
20	—	(60)	—	—

* Symbol "–", $K_m > 1000$ μM.
** Symbol "–", the reaction rate was less than 1% of that for natural substrate ($k_{cat} < 10^{-4}$ sec^{-1}).

substrates, but they effectively inhibit the enzyme activity: K_I values are 60 and 100 μM, respectively (Table 1). It should be noted that oxidized uridine derivative (**20**) [34], which exists in solution as a diastereomeric mixture of 1,4-dioxane derivatives (as illustrated in formula (2)) is also effectively bound by TP. It is likely that the 2′-hydroxyl group can be involved in nucleoside binding due to the formation of an additional hydrogen bond.

Therefore, based on kinetic data it is evident that the presence of 3′-hydroxyl group is essential for the phosphorolysis reaction to occur. The 2′-hydroxyl group enables more effective binding, but ribonucleosides are inhibitors of the reaction. The removal of the 5′-OH group has no effect either on the reaction rate or binding, and the presence of large substituents at the 5′-position decreases the reaction rate.

DISCUSSION

TP from *E. coli* contains two identical subunits with molecular weight of 45 kD each [1]. The cellular function of TP is to maintain thymidine content and refill it when necessary. Kinetic studies of bacterial TP demonstrate that the enzyme follows Michaelis kinetics and the reaction proceeds via the bi-bi mechanism, when the first step is phosphate binding, and deoxyribo-1-phosphate is the last product released from the catalytic center [39]. The detailed structure of *E. coli* TP obtained by X-ray (resolution 2.8 Å) was first presented in 1990. It was shown that each subunit contains a large domain formed by α-helices and β-sheets (so-called αβ-domain), and a small α-domain separated from the main one by the active site pocket. The authors obtained the structure of the enzyme

complex with thymidine and sulfate. They determined that the active center has two binding sites: nucleoside is bound in a small α-domain, whereas phosphate in bound in a large αβ-domain (the distance between the nucleoside and phosphate is approximately 9 Å), found the main amino acids involved in binding, and concluded that discrimination of ribonucleosides is associated with steric effects [42].

Further studies of the TP structure demonstrated that there are certain differences in the conformation of the active site before and after ligand binding. Three TP structure have been obtained: two without phosphate, and one for a complex with phosphate. Upon their comparison, it was found that after phosphate binding the αβ-domain is twisted by 9° relative to the α-domain. Correlating these data with the known structure (open conformation) and molecular dynamics computer simulation studies, the authors hypothesized domain movement and closure of the active site. The distance of 9 Å is too large for nucleophilic attack; therefore, the two domains should be brought in proximity (so-called closed conformation) to ensure proper positioning of phosphate and thymidine: down to 3.3 Å according to calculations [39].

Two other structures, crystallographic [43] and model [36], confirmed the hypothesis of a closed active center. The first structure is *B. stearothermophilus* PyNP in a complex with pseudouridine and phosphate obtained with resolution of 2.1 Å [43]. Compared to previous structures of *E. coli* TP, in this case (PyNP) the domains are in closer proximity (Table 2), and the active site is even more tightly closed. The substrate is localized in one of the subunits, and the pseudouridine heterocyclic ring (contrary to ribose ring) is well seen on electron density maps. The fact that PyNP is homologous to TP of *E. coli* and has the same tertiary structure (a homodimer) and a molecular weight of 92 kD (comparable with that of TP), allows considering this enzyme as a possible model for illustration and interpretation of kinetic data. The second structure is a model obtained by the molecular dynamics simulation [36] and shows an enzyme with a completely closed active site as a result of significant conformational changes.

Based on X-ray data for the TP complex with thymidine [39] and the molecular dynamics simulation [37], it is assumed that the formation of enzyme–substrate complex is driven by the interaction of amino acid residues Agr178, Lys190, and His85 with carbonyl groups C_4=O, C_2=O, Ser186 with HN_3, and Thr123 and Ser113 with phosphate. It is believed that the domain movement that starts with the formation of a complex leads to dramatic closing movement of the His85 imidazole ring and thymidine C_2=O. As a result of this closing movement, a carbonyl group becomes protonated, glycoside bond becomes weakened, and phosphate nucleophilic attack on the carbon cation is prepared [37].

To illustrate the results of kinetic measurements, the structures of thymidine and its modified analogs (HyperChem) were placed in the active center of known enzyme structures (using computer program Protein Viewer). TP structures in open conformation in a complex with sulfate were not a suitable model for illustration of the results, since they represented the enzyme during different stages of domain movement: the most closed conformation, according to authors' opinion, represents the partially closed active site [39]; closed conformation with pseudouridine could not be used for modeling due to the location of side radicals of some amino acids, which did not allow for substrate positioning because of steric hindrance. The use of completely closed enzyme conformation, obtained by molecular dynamics (MD) simulation (coordinates were kindly provided by Dr. Steven Rick, New Orleans University [36]) allowed positioning the substrate in the enzyme's active site and explaining the kinetic data. The distances between amino acid C_α atoms of the domains in open (2TPT.pdb), closed with pseudouridine (1BRW.pdb), and completely closed (MD) conformations are presented in Table 2.

Upon substrate positioning, we performed steric analysis, which demonstrated that the space around the sugar residue in a completely closed (MD) conformation is significantly restricted due to the proximity of the pro-

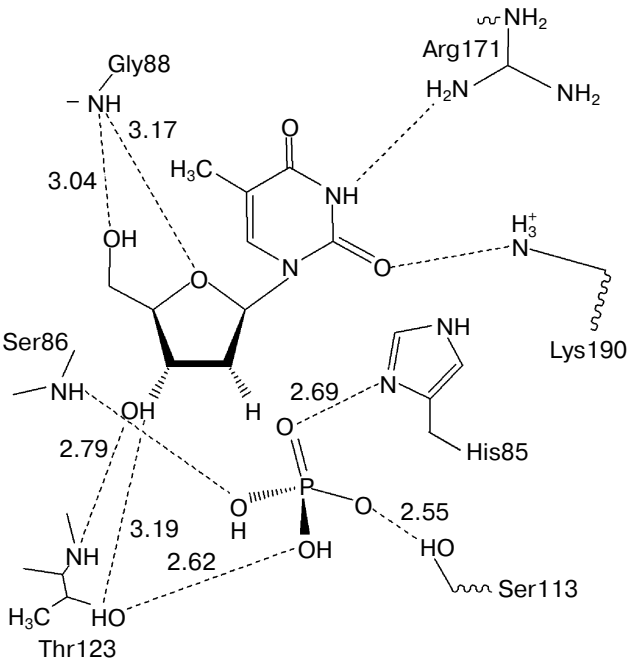
Table 2. Comparison of distances between C_α atoms of conservative residues of two domains in the TP active site pocket and distances between the phosphorus atom in the PO_4^{3-} anion and C_1' (distances were determined using the TURBO-FRODO program [23])

File name	Enzyme source	Amino acid		C_α–C_α, Å (P-C_1', Å)
		α-domain	α/β-domain	
2TPT.pdb [39]	*E. coli*	Ser186	Ser86	12.64 (9.0 [39])
1BRW.pdb [43]	*B. stearothermophilus*	Ser183	Ser83	11.3 (5.0)
tp_rot.pdb (MD) [36]	*E. coli*	Tyr186	Ser86	10.70 (4.5)

tein main chain. Then, using the TURBO-FRODO program [23], we analyzed possible contacts and hydrogen bonds between amino acids and the thymidine residue. As a model we used a closed conformation structure [36] (see Scheme below).

The fact that any modification of nucleoside sugar moiety (removal, replacement of OH-group, or introduction of additional groups at 3′- and 5′-position) has no or little effect on binding, but is significant for the reaction, indicated that ligand binding in the enzyme active site pocket can be accounted only for hydrogen bonds, and that 3′-OH (but not 5′-OH) plays a key role in the formation of enzyme–substrate complex in closed conformation and in occurrence of the reaction. In the proximity of 3′-hydroxyl group there is the main chain, and thus the formation of a hydrogen bond with the nitrogen atom of Thr123 is possible (Scheme). The formation of hydrogen bond (2.5 Å) with a nitrogen atom of His85 residue is possible upon the presence of 2′-hydroxyl group in the ribose ring (ribonucleosides). It is plausible that due to this additional bond, the affinity of the nucleoside for the enzyme is increased (compared to natural substrate), and, as a result, the interaction constant is decreased (K_I in the case of ribonucleosides). On the other hand, the formation of a hydrogen bond prevents His85 from being involved in protonation of C_2=O and inhibits the reaction.

The main conclusions made based on steric analysis and analysis of hydrogen bonds between the nucleoside and the active site amino acids in the closed enzyme conformation are the following. As soon as the nucleoside is bound in the TP active site due to hydrogen bonds of a heterocyclic base, it triggers the movement of the α-domain, which in turn results in dramatic closing movement of the nucleoside and phosphate; it is probable that this movement continues until the hydrogen bond between 3′-OH and HN-group of the main chain (Thr123) and phosphate oxygen atoms is formed. By all appearances, the formation of this bond is a final step in the association of enzyme–substrate complex that brings the nucleoside and phosphate into proper positions for nucleophilic attack.



Scheme for formation of enzyme–substrate complex: possible hydrogen bonds (designated by dashed lines) and contacts in closed conformation of *E. coli* TP (based on the coordinates from tp_rot.pdb [36])

This work was supported by the Russian Foundation for Basic Research (grant 04-04-49615-a) and Russian Academy of Sciences (program "Molecular and Cell Biology").

REFERENCES

1. Pugmire, M. J., and Ealick, S. E. (2002) *Biochem. J.*, **361**, 1-25.
2. Krenitsky, T. A., Koszalka, G. W., and Tuttle, J. V. (1981) *Biochemistry*, **20**, 3615-3621.
3. Utagawa, T. (1999) *Mol. Catalysis B: Enzymatic*, 215-222.
4. Barai, V. N., Zinchenko, A. I., Eroshevskaia, L. A., Kalinichenko, E. N., Kulak, T. I., and Mikhailopulo, I. A. (2002) *Helvetica Chimica Acta*, **85**, 1893-1900.
5. Upson, R. H., Haugland, R. P., Malek-zade, M. N., and Haugland, R. P. (1996) *Analyt. Biochem.*, **234**, 41-45.
6. Neizwicki, J., Kouni, M. H., Chu, S. H., and Cha, S. (1983) *Biochem. Pharmacol.*, **32**, 399-415.
7. Matsushita, S., Nitanda, T., Furukawa, T., Sumizawa, T., Tani, A., Nishimoto, K., Akiba, S., Miyadera, K., Fukushima, M., Yamada, Y., Yoshida, H., Kanzaki, T., and Akiyama, S. (1999) *Cancer Res.*, **59**, 1911-1916.
8. Focher, F., Ubiali, D., Pregnolato, M., Zhi, C., Gambino, J., Wright, G. E., and Spadari, S. (2000) *J. Med. Chem.*, **43**, 2601-2607.
9. Lieken, S., Bilsen F., De Clercq, E., Priego, E. M., Camarasa, M. J., Perez-Perez, M. J., and Balzarini, J. (2002) *FEBS Lett.*, **10**, 83-88.
10. McNally, V. A., Gbaj, A., Douglas, K. T., Stratford, I. J., Jaffar, M., Freeman, S., and Bryce, R. A. (2003) *Bioorg. Med. Chem. Lett.*, **13**, 3705-3709.
11. Price, M. L., Guida, W. C., Jackson, T. E., Nydick, J. A., Gladstone, P. L., Juarez, J. C., Donate, F., and Ternansky, R. J. (2003) *Bioorg. Med. Chem. Lett.*, **13**, 107-110.
12. Liekens, S., Hernandez, A.-I., Ribatti, D., de Clercq, E., Camarasa, M. J., Perez-Perez, M. J., and Balzarini, J. (2004) *J. Biol. Chem.*, **279**, 29598-29605.
13. Reigan, P., Edwards, P. N., Gbaj, A., Cole, C., Barry, S. T., Page, K. M., Ashton, S. E., Luke, R. W., Douglas, K. T., Stratford, I. J., Jaffar, M., Bryce, R. A., and Freeman, S. (2005) *J. Med. Chem.*, **48**, 392-402.
14. Zimmerman, M., and Seidenberg, J. (1964) *J. Biol. Chem.*, **239**, 2618-2621.
15. Razzel, W. E., and Casshyp, P. (1964) *J. Biol. Chem.*, **239**, 1789-1793.

16. Schwartz, M. (1978) *Meth. Enzymol.*, **51**, 442-445.
17. El Kouni, M. H., el Kouni, M. M., and Naguib, F. N. (1993) *Cancer Res.*, **53**, 4738.
18. Nakayama, C., Wataya, Y., Meyer, R. B., Jr., Santi, D. V., Saneyoshi, M., and Ueda, T. (1980) *J. Med. Chem.*, **23**, 962-964.
19. Schinazi, R. F., Peck, A., and Sommadossi, J. P. (1992) *Biochem. Pharmacol.*, **44**, 199-204.
20. Kono, A., Hara, Y., Sugata, S., Matsushima, Y., and Ueda, T. (1984) *Chem. Pharm. Bull. (Tokyo)*, **32**, 1919-1921.
21. Zintchenko, A. I., Eroshevskaya, L. A., Barai, V. N., and Mikhailopulo, I. A. (1987) *Nucleic Acids Symp. Ser.*, **18**, 137-140.
22. Esipov, R. S., Gurevich, A. I., Chuvikovsky, D. V., Chupova, L. A., Muravyova, T. I., and Miroshnikov, A. I. (2002) *Protein Expr. Purif.*, **24**, 56-60.
23. Russel, A., and Cambillou, C. (1991) *Silicon Graphics Geometry Partner Directory*, Silicon Graphics, Mountain View, CA, USA, p. 81.
24. Wang, Y., and Hogenkamp, H. P. C. (1977) *Carbohydr. Res.*, **59**, 449-457.
25. Verheyden, J. P. H., and Moffatt, J. G. (1972) *J. Org. Chem.*, **37**, 2289-2299.
26. Tsuji, T., and Takenaka, K. (1987) *Nucleosides Nucleotides*, **6**, 575-580.
27. Bannwarth, W. (1988) *Helv. Chim. Acta*, **71**, 1517-1527.
28. Padyukova, N. Sh., Fomicheva, M. V., Mikhailov, S. N., and Yanta-Lipinski, M. (1990) *Bioorg. Khim.*, **16**, 668-673.
29. Panova, N. G., Shcheveleva, E. V., Alexeev, C. S., Mukhortov, V. G., Zuev, A. N., Mikhailov, S. N., Esipov, R. S., Chuvikovsky, D. V., and Miroshnikov, A. I. (2004) *Mol. Biol. (Moscow)*, **38**, 770-776.
30. Giller, S. A., Zhuk, R. A., and Lidak, M. Yu. (1967) *Doklady AN SSSR*, **176**, 332-335.
31. Horwitz, J. P., Chua, J., da Rooge, M. A., Noel, M., and Klundt, I. L. (1966) *J. Org. Chem.*, **3**1, 205-211.
32. Horwitz, J. P., Chua, J., and Noel, M. (1964) *J. Org. Chem.*, **29**, 2076-2078.
33. Horwitz, J. P., Chua, J., Urbanski, J. A., and Noel, M. (1964) *J. Org. Chem.*, **28**, 942-944.
34. Yermolinskii, B. S., and Mikhajlov, S. N. (2000) *Bioorg. Khim.*, **26**, 483-504.
35. Dixon, M., and Webb, E. (1982) *Enzymes* [Russian translation], Mir, Moscow.
36. Rick, S. W., Abashkin, Y. G., Hilderbrandt, R. L., and Burt, S. K. (1999) *Proteins*, **37**, 242-252.
37. Mendieta, J., Martin-Santamaria, S., Priego, E. M., Balzarini, J., Camarasa, M. J., Perez-Perez, M. J., and Gago, F. (2004) *Biochemistry*, **43**, 405-414.
38. Komatsu, H., and Awano, H. (2002) *J. Org. Chem.*, **67**, 5419-5421.
39. Pugmire, M. J., Cook, W. J., Jasanoff, A., Walter, M. R., and Ealick, S. E. (1998) *J. Mol. Biol.*, **281** 285-299.
40. Liebonaska-Lis, E. (1989) *Nucleosides Nucleotides*, **8**, 383-405.
41. Oivanen, H., Hovinen, J., Lehikoinen, P., and Lonberg, H. (1993) *Trends Org. Chem.*, **4**, 397-411.
42. Walter, M. R., Cook, W. J., Cole, L. B., Short, S. A., Koszalka, G. W., Krenitsky, T. A., and Ealick, S. E. (1990) *J. Biol. Chem.*, **265**, 14016-14022.
43. Pugmire, M. J., and Ealick, S. E. (1998) *Structure*, **6**, 1476-1479.