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Molecular Characterization, Heterologous Expression and Kinetic Analysis of Recombinant *Plasmodium falciparum* Thymidylate Kinase

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The gene encoding for thymidylate kinase from *Plasmodium falciparum* was obtained by PCR and expressed in *Escherichia coli* and the enzyme was investigated as a possible new drug target. The enzyme is a homodimer exhibiting maximal kinase activity over a wide pH range of 7–9 and is characterized by marked stability. Compared with the human enzyme, the recombinant *P. falciparum* TMP kinase showed a broader spectrum of substrate specificity. The enzyme not only phosphorylates dTMP and dUMP but can also tolerate the bulkier purines dGMP, GMP and dIMP. Initial velocity studies showed that the K_m values for TMP and dGMP are 22 and 30 μM, respectively. The turnover number $k_{cat(TMP)}$ was found to be 3.4 s^{-1}, a value indicating the higher catalytic efficiency of the plasmodium enzyme. From the present study, we suggest that the design of appropriate inhibitors especially purine based compounds could have a selective inhibitory effect on the parasite enzyme.

Key words: enzyme kinetics, malaria, *Plasmodium falciparum*, thymidylate kinase.

Abbreviations: NMP, nucleoside monophosphate; PfTMK, *Plasmodium falciparum* thymidine monophosphate kinase; rPfTMK, recombinant *Plasmodium falciparum* thymidine monophosphate kinase; TMK, thymidine monophosphate kinase.

Malaria is thought to be a world-wide problem and it is considered an obligate parasitic disease in certain areas. More than one third of the world's population (about 2 billion people) live in malaria-endemic areas, and 1 billion people are estimated to carry parasites at any one time (*1*). Every year, about 500 million people become infected and there are more than 2 million deaths (*2*). Lack of an effective vaccination and the spread of drug resistance necessitate the development of new drug targets. In the development of antimalarial drugs, it may be suitable to select targets from pathways present in the parasite but absent in humans. Nevertheless, even if a target was common to both, parasite and host, slight structural differences could enhance the optimization of a new drug (*3*). A difference in only one amino acid residue between human and Plasmodium *S*-adenosyl-L-homocysteine hydrolase led to the discovery of highly selective inhibitor drugs (*4–6*). The rational approach to drug development starts with the identification of new molecular targets critical for the life cycle of the parasite. Enzymes of nucleic acid metabolism seem to be important candidates due to their indispensable biological role. Of particular interest are the nucleoside monophosphate (NMP) kinases, which play important roles in the synthesis of DNA and RNA precursor nucleotides.

Thymidylate kinase [thymidine monophosphate kinase (TMK), ATP: TMP phosphotransferase, dTMP kinase, deoxythymidine monophosphate kinase, **EC 2.7.4.9**] belongs to the NMP kinase super-family and is critical for the synthesis of TTP. It catalyzes the reversible phosphorylation of dTMP to its diphosphate form, dTDP, in the presence of the divalent cation Mg^{2+} with ATP as the preferred phosphate donor (*7*). TMKs have medical and therapeutic importance for the activation of the nucleoside analogue prodrugs used as antiviral and anticancer drugs (*8*). We expect that thymidylate kinase will be a potential antimicrobial drug target since its inhibition modulates the synthesis of nucleotides, which are indispensable for any organism.

Detailed knowledge of the enzymatic properties of recombinant *Plasmodium falciparum* thymidine monophosphate kinase (rPfTMK) has not yet been determined. We undertook cloning, heterologous expression, detailed characterization, substrate specificities and enzyme kinetics studies in an attempt to acquire essential knowledge for the development of new inhibitory drugs. In this article, we have shown the atypical functional parameters of rPfTMK distinguishing it from other TMKs. In addition, we anticipate that *Plasmodium falciparum* thymidine monophosphate kinase (PfTMK) could serve as a potential new drug target for the design of highly selective drugs.

MATERIALS AND METHODS

Materials—Escherichia coli JM109, Sephacryl S200HR, and ova albumin used for the calibration of the gel filtration column (44 kDa) were obtained from

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GE Healthcare. AccuPrime Pfx polymerase was obtained from Invitrogen. Talon metal affinity resin was obtained from Clontech (Palo Alto, CA). The vector pQE-30 was obtained from Qiagen. NMPs, nucleoside triphosphates, phosphoenolpyruvate and molecular weight markers for the calibration of the gel filtration column—alcohol dehydrogenase (150 kDa) and cytochrome c (12.4 kDa)—were obtained from Sigma. Lactate dehydrogenase and pyruvate kinase were obtained from Wako Pure Chemicals. NADH was obtained from Oriental Yeast (Tokyo, Japan). Protein concentrations were determined by using a protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

Database Search and Sequence Comparisons—Sequences of TMKs from different bacterial and eukaryotic sources were used individually to identify similar sequences in a *Plasmodium falciparum* genome database by using the Basic Local Alignment Search Tool available at http://www.ncbi.nlm.nih.gov/blast/. High similarity hits were used for multiple sequence alignment with ClustalW available at http://www.ebi.ac.uk/. Alignment was refined manually using GeneDoc version 2.62. The 3D structures of *P. falciparum* TMK was modelled using the First Approach mode available at the SWISS-MODEL server (http://www.expasy.org/swissmod/) and the structures were viewed by using CueMol (R. Ishitani, CueMol: Molecular Visualization Framework; cuemol.sourceforge.jp). Putative domains were searched by the domain prediction program available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml.

Molecular Cloning—Plasmodium falciparum—FCR-3 strain cDNA was used as a template (obtained by RT-PCR of total cell RNA). The gene coding for PfTMK was obtained by PCR using a pair of primers—forward (5′-CACGGATCCATGACTGATGATAAAAAAAAAGG-3′) and reverse (5′-CGCAAGCTTTTATGACCACAAAAAATT AAATTCTTC-3′)—with *BamHI* and *HindIII* restriction sites (underlined). The product of PCR was digested with *BamHI* and *HindIII* and subcloned into pQE-30 vector predigested with the same enzymes. The resulting plasmid was termed pQE30PfTMK. Competent JM109 *E. coli* cells were transformed with the plasmid pQE30PfTMK.

Expression of rPfTMK in E. coli—The recombinant *E. coli* cells were grown overnight in an LB medium containing 50 μg/ml ampicillin. The culture was diluted to 1 : 100 with the same fresh medium and cells were grown at 37°C to mid-log phase ($D_{600} = 0.6$). Induction of expression was carried out by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 1 mM and cell growth was continued at 37°C for 4 h. Cells from 2 l of culture were harvested by centrifugation at 5,000*g* for 15 min and then stored at −20°C until use. To obtain the cell extract, cells were lysed in an extraction buffer (25 mM Tris–HCl buffer pH 7.2 containing 150 mM NaCl) and disintegrated by sonication for 40 s (three cycles with 3 min intervals). The lysate was centrifuged at 16,000*g* for 15 min at 4°C and the precipitate is discarded.

Affinity Chromatography—The hexahistidine-tagged rPfTMK was purified from the soluble cell extract by using talon metal affinity resin. After binding and washing, the protein was eluted using a stepwise imidazole gradient in which the column was preliminary washed with 10 column volumes of extraction buffer containing 10 mM imidazole. Further washing with one column volumes of 50 and 100 mM imidazole was adopted. Highly purified protein was eluted by 200 mM imidazole (three column volumes). Protein concentrations were determined by the Bradford method (*9*).

Analytical Gel Filtration Chromatography—The oligomeric state of rPfTMK was determined in a sephacryl S200HR column (90 × 1.6 cm) pre-equilibrated with the extraction buffer at a flow rate of 0.5 ml/min. Molecular weight standards were independently chromatographed on the same column. The relative elution of rPfTMK was compared with that of the standard markers.

Thymidylate Kinase Assay—The TMP kinase activity was measured spectrophotometrically using an enzyme coupling assay (*10*) and by HPLC (*11*). In the spectrophotometric assay, the standard assay of 1 ml contained the following: 50 mM Tris–HCl pH 7.2, 0.5 mM phosphoenolpyruvate, 40 mM KCl, 2 mM $MgCl_2$, 0.15 mM NADH, 2U pyruvate kinase, 2U lactate dehydrogenase and various concentrations of NMP and ATP. The reaction was initiated by the addition of the recombinant enzyme. The decrease in absorbance at 335 nm was monitored at 30°C. One unit of enzyme activity is defined as the amount of enzyme catalyzing the production of 1 μmol nucleoside diphosphate/minute under the above conditions. The results are compared with those for a control experiment using purified extracts from *E. coli* transformed with the vector without any inserts.

HPLC assay was used to characterize rPfTMK activity and to avoid the disadvantages of the coupled enzyme assay. The activity of TMKs as a function of pH, metal requirements as well as the assay for inhibitors is complicated by sensitivity of the coupling enzymes. To validate the HPLC assay, both ATP and TMP were tested using the direct assay and enzyme coupling assay to demonstrate that their apparent Michaelis constants are the same. The reaction was carried out in 1 ml final volume of a solution of 50 mM Tris–HCl, pH 7.2, 40 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol and variable concentrations of ATP or TMP. The buffer used for elution is 50 mM sodium phosphate, pH 6.5, 2.5% (v/v) ethanol, 40 mM terabutylammonium bromide. The concentration of separated nucleotides is monitored at 260 nm using Mightysil C18, 5 μm column (Kanto chemicals Co.).

RESULTS

Putative Domains, Sequence Comparison and Molecular Modelling—Screening of the *P. falciparum* databases revealed that thymidylate kinase is likely to be functionally different from the human enzyme. Analysis of the amino acid sequence using the domain prediction program revealed that the plasmodium enzyme possesses a single NMP binding domain with properties different from that of human TMK. In addition to the thymidylate kinase, it was predicted that PfTMK can possess adenylate kinase and related kinases properties (Supplementary Fig. 1).

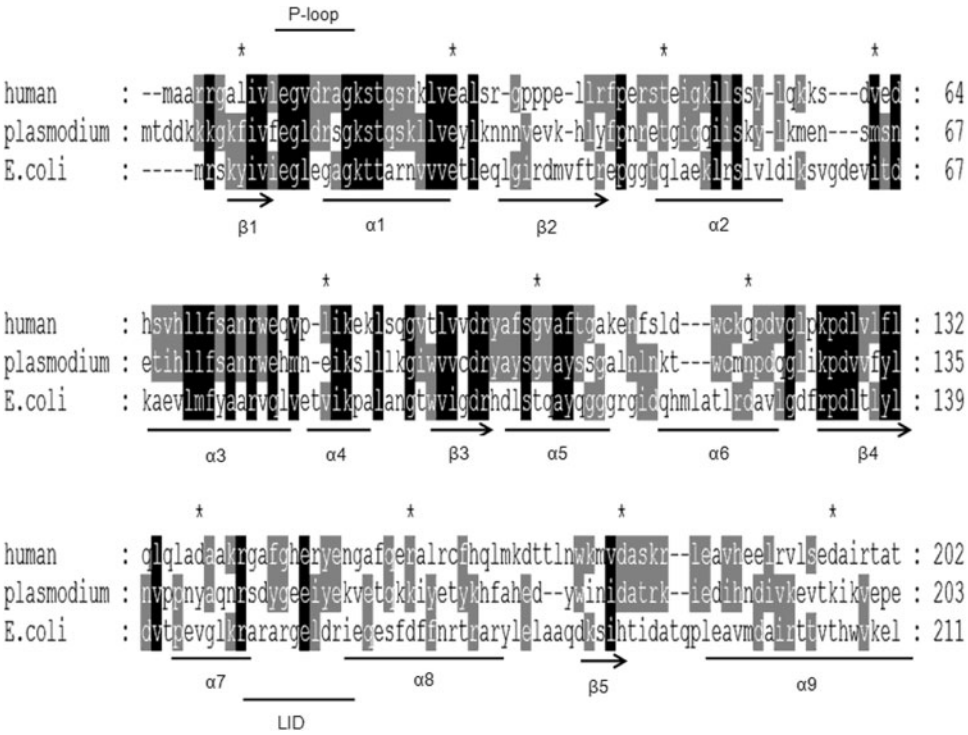

Fig. 1. **Amino acid sequence alignment in human (39% identity), *E. coli* (28% identity) and *P. falciparum* TMKs.**

The comparison of PfTMK with TMKs of humans, mouse, *Saccharomyces cerevisiae* and *E. coli* revealed that PfTMK shows 27–41% identity. The phosphate binding loop (P-loop) with the conserved sequence GXXXXGK(S/T) (where X is any amino acid) is found in residues 15–22 (Fig. 1). Residues 98–107 are found to align perfectly with the general TMP binding motif DR(Y/F/H)XXSXXA(F/Y). TMKs are classified into two types, according to the position of the active-site arginine residues (*12*). Type I TMKs have an invariant arginine residue in the P-loop and lack such a residue in the LID domain, *e.g*. human and yeast TMKs. In contrast, type II TMKs lack basic residues in the P-loop and contain an arginine in the LID domain that is not strictly conserved, *e.g*. *E. coli* TMK. From the alignment data, we can deduce that the *P. falciparum* sequence is closer to eukaryotic enzymes and could be classified as type I TMK.

According to our model of PfTMK structure, the general folding pattern and the structure of the active site are almost identical to those of human TMK. However, at the active site of PfTMK, Tyr43, Tyr107 and Ser108 replace human TMK Arg41, Phe105 and Thr106, respectively. Careful examination of the PfTMK model revealed the replacement of some residues near to the proposed active site, particularly at the junction of the α2–α3, α5–α6 and α8 loops where the presence of bulky residues, such as His66, Lys109 and Phe156 are observed in human TMK. In contrast, the previously mentioned residues are replaced by smaller residues Glu68, Ala111 and Thr158 in PfTMK (Supplementary Figs 2 and 3).

Table 1. **Summary of purification of rPfTMK from *E.coli*.**[a]

Fraction	Total volume (ml)	Activity (units/ml)	protein (mg/ml)	Specific activity (units/mg)	Relative purity
Cell extract	30	2.6	10	0.26	1
Metal affinity	3	22.5	1.5	15	57.6

[a]Activity was measured using TMP as a substrate.

Over Expression and Purification of rPfTMK—A cell extract containing the N-terminal hexahistidine-tagged rPfTMK was purified by talon metal affinity chromatography resin (Table 1). In order to obtain a highly purified product, a stepwise gradient of imidazole wash was adopted (up to 100 mM concentration) and elution was carried out with 200 mM concentration. Although most of the rPfTMK was lost by this procedure, we obtained a highly purified product after one step of purification. In addition, we ran a control experiment (extract from JM109 *E. coli* cells transformed with pQE-30 vector and purified by the same procedure). Expectedly, weak thymidylate kinase activity is detected in the early eluted fraction with no detectable activity in the latter fractions. The purity of products was assessed by SDS–polyacrylamide gel electrophoresis stained with Coomassie brilliant blue. The band observed at 24 kDa was further confirmed by western blotting using an anti-hexahistidine-tag antibody. By gel filtration chromatography rPfTMK was eluted in a single peak at a MW calculated to be 50 kDa, which corresponds to a dimer.

Thymidylate Kinase Activity—Under standard assay conditions, the reaction appeared to be linear for 30 min.

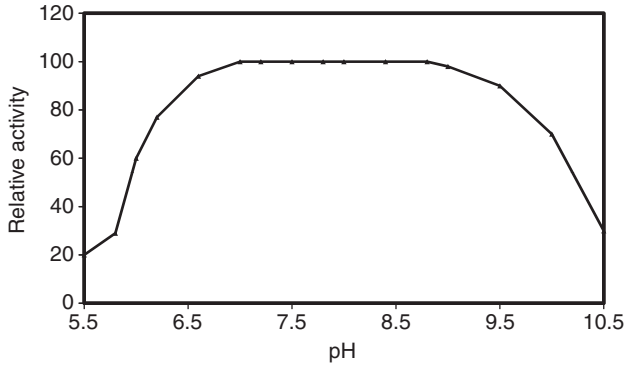

Fig. 2. **Effect of pH on thymidylate kinase activity.** Activity was measured by HPLC separation by monitoring the increase in TDP concentration.

The activity of rPfTMK was sensitive to $MgCl_2$ and KCl concentrations. The variation of thymidylate kinase activity as a function of pH was examined (Fig. 2). To avoid the poor reactivity of coupling enzymes at extreme pH ranges, direct measurement of nucleotides by HPLC separation was adopted. The activity of rPfTMK was observed over the entire range of pH (*6–10*) with maximal activity between pH 7–9. The activity was markedly dropped below pH 6. rPfTMK, as other TMKs, showed high stability. No change was observed in the activity from the enzyme kept at –20°C for 2 months.

In order to estimate the stability of rPfTMK at different temperatures, aliquots of rPfTMK were incubated over a temperature range of 40–70°C for 10 min. The amount of residual activity was determined by the standard assay. The enzyme activity was decreased 30% at 60°C and completely lost after preincubation at 65°C.

The enzyme activity was decreased 15% in the presence of UMP, dUMP and dAMP in 5 mM concentration while no change is detected with other NMPs (AMP, CMP, dCMP, GMP and dGMP). EDTA at 4 mM caused 80% loss of the kinase activity which is completely lost at 5 mM concentration. This case is described as a non-specific mechanism of inhibition due to abrupt inhibition above a critical concentration. This was an expected effect since the enzyme is dependent on divalent cations for its activity. 2-Mercaptoethanol in concentrations of up to 5 mM did not have an effect on the reaction rates.

Substrate Spectrum and Specificity for Phosphate Donors—rPfTMK was found to have a unique specificity for phosphate acceptors deviating from the properties of known TMKs. The enzyme showed the highest specificity for dTMP and to a lesser extent dUMP. The most striking and unique feature of this enzyme is its ability to utilize the purine nucleosides GMP, dGMP and dIMP. The specific activities for GMP and dIMP were as low as 0.12 and 2.2 U/mg, respectively. However, the enzyme can utilize dGMP with very high efficiency, quite similarly to dTMP. No phosphorylation activity was detectable with AMP, dAMP, CMP, dCMP and UMP (Table 2).

Of the eight ribo and deoxyribonucleoside triphosphates tested (ATP, CTP, GTP, UTP, dATP, dGTP, dCTP and TTP), only ATP and dATP were the most active as phosphate donors. rPfTMK can use CTP, GTP, TTP and UTP to some degree (3–10%). No activity was observed with dCTP and dGTP (Table 3).

Table 2. **Activity of various nucleoside monophosphates as phosphate acceptors.**

Nucleoside monophosphate	Concentration (mM)	Activity (%)
TMP	1	100
dGMP	1	86
dUMP	10	43
dIMP	10	15
GMP	10	1.5
AMP/dAMP/CMP/dCMP/UMP	Up to 5[a]	N.D.[b]

[a]Substrate concentrations are 0.02, 0.1, 0.5, 1, 2 and 5 mM.
[b]N.D.: Activity was not detectable.
These values were obtained from the standard assay (as described in MATERIAL AND METHODS section).

Table 3. **Activity of various nucleoside triphosphates as phosphate donors.**

Nucleoside triphosphate	Concentration (mM)	Relative activity
ATP	2	100
dATP	2	64
GTP/CTP/dTTP/UTP	Up to 5	<10
dCTP/dGTP	Up to 5[a]	N.D.[b]

[a]Substrate concentrations are 0.02, 0.1, 0.5, 1, 2 and 5 mM.
[b]N.D.: Activity was not detectable.
These values were obtained from the standard spectrophotometric assay (as described in MATERIAL AND METHODS section).

Table 4. **Summary of the kinetic properties of recombinant PfTMK.**

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Specific activity (U/mg)
dTMP	22 ± 3.6	3.4 ± 0.14	154.5	15
dGMP	30.7 ± 7.2	2.9 ± 0.25	94.4	13
dUMP	780 ± 40.2	4.1 ± 0.4	5.2	6.5
dIMP	577 ± 28.5	0.44 ± 28.5	0.77	2.2

The enzyme kinetic constants were obtained from the standard assay (as described in MATERIALS AND METHODS section). The result is an average of at least three different experiments. The substrate concentration was varied (0.002–2 mM) in the case of dTMP and dGMP, while the dUMP and dIMP level was 0.01–10 mM. K_m and V_{max} values were calculated from Lineweaver–Burk plots.

Kinetic Characterization—For determination of kinetic parameters, the initial reaction rates were measured at different concentrations of substrates. rPfTMK can phosphorylate dTMP with a catalytic efficiency of $k_{cat}^{dTMP}/K_m^{dTMP} = 154\ s^{-1}mM^{-1}$, with $K_m^{dTMP} = 22\ \mu M$ and $k_{cat} = 3.4\ s^{-1}$ (Table 4). Interestingly, dGMP can serve as a substrate for rPfTMK, with kinetic parameters highly comparable to those of dTMP. The $k_{cat}^{dGMP}/k_{cat}^{dTMP}$ value was about 0.85, indicating the ability of rPfTMK to utilize either purine or pyrimidine nucleosides with very similar efficiency. The catalytic efficiencies for dUMP and dIMP were remarkably low: 29- and 200-fold lower than that of dTMP. The saturation curve for ATP indicated K_m^{ATP}

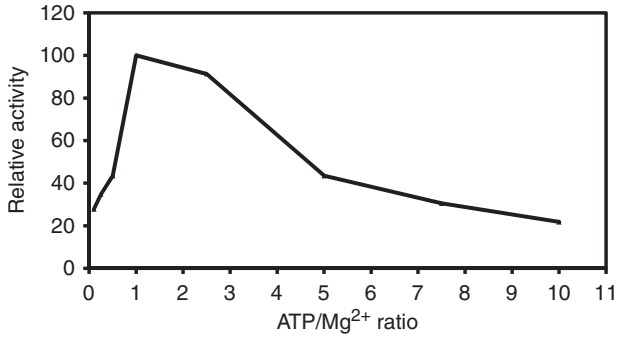

Fig. 3. **Effect of ATP:Mg^{2+} ratio on thymidylate kinase activity.** Equimolar concentration of ATP and Mg^{2+} is a marginal value for optimum activity.

of 218 μM with a turnover number of 3.4 s^{-1} at equimolar concentrations of ATP and Mg^{2+}. The thymidylate kinase activity was found to be sensitive to ATP/Mg^{2+} ratio (Fig. 3). The maximal activity was achieved at equimolar concentrations of ATP and Mg^{2+}. In the presence of excess amount of ATP, a marked reduction of activity was observed. Interestingly, no reduction of reaction rates was detected up to 20 mM ATP, if combined with Mg^{2+} in an equimolar concentration.

DISCUSSION

Thymidylate kinase has physiological and biomedical importance since it is essential for the formation dTDP. Inhibition of TMK has an impact on DNA and RNA synthesis in addition to the loss of many metabolic processes in cells regulated by TMK activity. Thus far, no information exists on the properties of *P. falciparum* thymidylate kinase. To better understand this enzyme, we characterized its biochemical properties, substrate specificities and enzyme kinetics.

The present results indicate that rPfTMK shares the majority of its properties with other TMKs. The conserved structure of the catalytic domains, the dimeric status, broad pH range, and most of the kinetic properties are all essentially the same. The k_{cat}^{dTMP} was found to be within the estimated range of known TMKs (5–45 μM). The turnover number k_{cat} of known TMKs was found to be within 0.4–2.4 s^{-1} (*13*–*16*), therefore, rPfTMK performs thymidylate kinase activity with higher catalytic efficiency.

The most remarkable and unique feature of rPfTMK is its ability to utilize both purine and pyrimidine nucleosides as substrates. In human TMK, the cavity in which the base binds is very tight to accommodate purine bases, and thus reduces specificity to pyrimidines (*17*, *18*). In spite of the high similarities between the two enzymes, the human enzyme possesses high substrate specificity and is devoid of any purine NMP kinase activity. It will be interesting to determine the mechanisms by which rPfTMK can tolerate both purine and pyrimidine NMPs in its active site. This will require resolution of the crystal structure of PfTMK with various substrates. Based on the model of structure of PfTMK we predict that few amino acid replacements may be the cause underlying the ability of rPfTMK to use purine NMPs. The presence of smaller amino acid residues near to the active site in PfTMK (which is replaced by bulkier one in human TK) may contribute to a larger and more flexible active site to accommodate the purine ring.

The phenomenon of being able to identify a range of structurally dissimilar substrates was an attractive finding in the field of enzymology and drug design. The known NMP kinases recognizing both dTMP and dGMP are the bacteriophage T4 deoxynucleotide kinase (*19*) and vaccinia virus TMK (*20*). Interestingly, the bacteriophage enzyme is similar to the fold of NMP kinases, with a different dimerization mode while the TMP binding site in vaccinia virus TMK model is almost identical in human enzyme with a single amino acid substitution. Moreover, several of the nucleoside kinases have diverse substrate specificities *e.g.* herpes virus thymidine kinase which can utilize both thymidine and deoxyguanosine (*21*) and the Drosophila deoxynucleosides kinase which has the broadest spectrum that includes all deoxynucleosides (*22*).

In our previous work, we demonstrated that dGMP is a poor substrate for *P. falciparum* guanylate kinase (*23*). This finding and our present finding, which revealed that rPfTMK can utilize dGMP as a substrate with high efficiency, will contribute to knowledge on the nucleic acids metabolism in *P. falciparum*.

The extended substrate specificity of herpes virus thymidine kinase has provided the basis of selective antiherpetic therapy (*21*). Similarly, we would expect that inhibitors based on modifications of purine nucleosides will be highly selective against PfTMK. Therefore, further investigations will be required to resolve the 3D structure of PfTMK with dTMP and dGMP, which will provide the basis for designing the appropriate inhibitors.

Supplementary data are available at *JB* Online.

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