

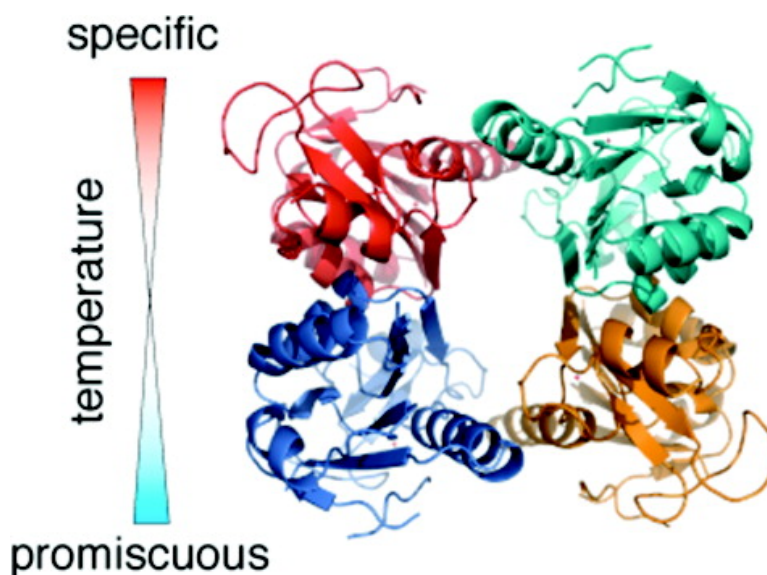
Communication

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*J. Am. Chem. Soc.*, 2007, 129 (28), 8714-8715 • DOI: 10.1021/ja0734391 • Publication Date (Web): 26 June 2007

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## Exploiting Temperature-Dependent Substrate Promiscuity for Nucleoside Analogue Activation by Thymidine Kinase from *Thermotoga maritima*

Stefan Lutz,\* Joseph Lichter, and Lingfeng Liu

Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, Georgia 30322

Received May 15, 2007; E-mail: sal2@emory.edu

Enzymes from extremophilic organisms offer many interesting opportunities for applications in organic synthesis and biotechnology.<sup>1,2</sup> They often exhibit tolerance for denaturants and organic solvents, as well as operate at extreme temperatures, pH, and pressures. Enzymes from thermophilic and hyperthermophilic organisms are particularly attractive as increased stability and function at elevated temperature are desirable features for biocatalysis.

The temperature dependency of enzyme catalysis has been studied for several thermophilic biocatalysts, typically showing declining activity with decreasing temperature.<sup>3,4</sup> This effect has been attributed to increasing rigidity of proteins at lower temperature. In contrast, little is known about how temperature might affect substrate specificity in enzyme catalysis. As part of our investigation of deoxynucleoside kinases (dNKs) for the phosphorylation of nucleoside analogue (NA) prodrugs, we have isolated a novel thymidine kinase from the hyperthermophilic eubacterium *Thermotoga maritima* (TmTK) and explored the effects of temperature on the substrate specificity toward natural and unnatural nucleosides.

The gene for TmTK was isolated from *T. maritima* genomic DNA and heterologously overexpressed in *E. coli*. Following purification of the enzyme to homogeneity, crystallography confirmed its high similarity to human TK1, both in tertiary and quaternary structure.<sup>5</sup> We are now reporting the catalytic performance of TmTK with various phosphoryl donors, as well as natural 2'-deoxyribonucleosides and NAs as phosphoryl acceptors at 37 and 82 °C, *T. maritima*'s normal growth temperature (Table 1 and S1). Initial kinetic measurements at 37 °C indicate low donor specificity with a preference for ATP. In contrast, the enzyme is highly specific in regards to the nucleobase of the phosphoryl acceptor. Consistent with other members of the TK1-like family, TmTK phosphorylates only thymine and uracil-carrying substrates. The strict preference results from specific hydrogen-bonding interactions between the pyrimidine moiety of the substrate and the enzyme's lasso region.<sup>5,6</sup>

At 82 °C, TmTK's catalytic rates, apparent binding constants, and cooperativity data for T and ATP closely resemble its mesophilic counterparts at their respective optimal temperatures (Table 1). Contrary to many hyperthermophilic enzymes, TmTK maintains significant activity at suboptimal temperatures. At 37 °C, the  $k_{\text{cat}}/K_{\text{M}}$  values for T and ATP are reduced by only 5.8- and 1.8-fold, respectively. We also noticed the loss of ATP cooperativity in the homotetramer. More interestingly, the kinetic measurements with AZT at both temperatures showed the opposite trend. While AZT is phosphorylated with 20-fold lower efficiency than T at 82 °C, its  $k_{\text{cat}}/K_{\text{M}}$  value at 37 °C matches the natural substrate. More so, the enzyme is a more efficient catalyst for AZT phosphorylation at 37 °C than at 82 °C, showing a 2.5-fold improvement in  $k_{\text{cat}}/K_{\text{M}}$  at the lower temperature. Hence, it appears that the enzyme can discriminate against unnatural substrates under physiological conditions at 82 °C, yet it shows broader substrate tolerance at 37 °C.

**Table 1.** Kinetic Parameters for TmTK Substrates at 37 and 82 °C

substrate	$K_{\text{M}}$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}} \times 10^4$ ( $\text{M}^{-1} \text{s}^{-1}$ )	relative efficiency	Hill coeff
T(37°C)	0.5 ± 0.2	0.3 ± 0.08	60	1	0.98
T(82°C)	1.0 ± 0.15	3.5 ± 0.3	350	5.8	1.1
ATP(37°C)	40 ± 12	0.4 ± 0.04	1.0	1	1.13
ATP(82°C)	187 ± 21 ( $K_{0.5}$ )	3.5 ± 0.3	1.8	1.8	2.4
AZT(37°C)	0.65 ± 0.2	0.29 ± 0.03	45	1	-
AZT(82°C)	8.7 ± 3	1.6 ± 0.29	18	0.4	-

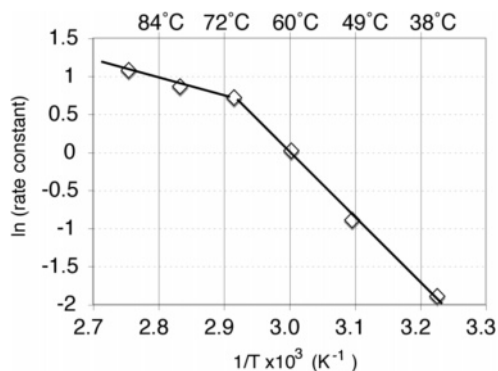
**Table 2.** Kinetic Parameters for Nucleoside Analogues at 37 °C<sup>a</sup>

substrate	$K_{\text{M}}$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}} \times 10^4$ ( $\text{M}^{-1} \text{s}^{-1}$ )	relative activity	preferred conformation
dU	1.5 ± 1	0.7 ± 0.1	48	0.8	C <sub>2'</sub> -endo
U	>1000	nd			C <sub>3'</sub> -endo
5-FdUrd	1 ± 0.1	0.74 ± 0.05	74	2.5	C <sub>2'</sub> -endo
ddT	30 ± 5	0.33 ± 0.04	1.1	1.1	C <sub>2'</sub> -endo
ddU	>1000	nd			C <sub>3'</sub> -endo
AZT	0.65 ± 0.2	0.29 ± 0.03	45	1	C <sub>3'</sub> -exo
d4T	75 ± 5	0.41 ± 0.02	0.54	1.4	planar
DOT	114 ± 15	0.29 ± 0.02	0.25	1	C <sub>3'</sub> -endo
L-FMAU	132 ± 4	0.5 ± 0.02	0.38	1.7	C <sub>2'</sub> -endo
L-T	>1000	nd			C <sub>2'</sub> -endo

<sup>a</sup> (nd) = not determined as  $v_{\text{max}}$  was not reached at 1000  $\mu\text{M}$  substrate; dU = 2'-deoxyuridine; U = uridine; ddT = 2',3'-dideoxythymidine; ddU = 2',3'-dideoxyuridine; 5FdUrd = 5-fluoro-2'-deoxyuridine; AZT = 2',3'-dideoxy-3'-azidothymidine; d4T = 2',3'-dideoxy-2',3'-didehydrothymidine; L-FMAU = 2'-fluoro-5-methyl- $\beta$ -L-arabinofuranosyluracil; L-T = L-thymidine; DOT = dioxolane thymidine.

Such temperature-dependent substrate promiscuity of extremozymes has not been reported previously but could be of fundamental interest in biocatalysis, enzyme engineering, and protein evolution.

To further investigate this phenomenon, we expanded our study to other NAs. While experiments at elevated temperatures were limited by the availability of tritiated substrates with sufficient specific activity, kinetic measurements with unlabeled NAs using a spectrophotometric coupled-enzyme assay at 37 °C confirmed the broad tolerance of TmTK for nucleosides with modified ribose moieties (Table 2). Differences in catalytic performance arise from variation in the  $K_{\text{M}}$  values and correlate well with the preferred sugar conformation of the substrate. Crystallography and NMR spectroscopy, as well as studies with nucleosides in locked conformations, have established the preferred C<sub>2'</sub>-endo sugar puckering of nucleoside bound to dNKs.<sup>7-9</sup> TmTK follows the same trend, favoring substrates with C<sub>2'</sub>-endo and C<sub>3'</sub>-exo sugar puckering over ribose with a C<sub>3'</sub>-endo conformation. In addition, unfavorable steric effects are likely responsible for the decreased binding affinity of NAs carrying the L-isomer of 2'-deoxyribose or a ribose moiety. Similarly,  $K_{\text{M}}$  values for 2',3'-dideoxynucleosides increase due to missing stabilizing hydrogen-bonding interactions. In summary, the sugar puckering accounts for the differences in  $K_{\text{M}}$  values at



**Figure 1.** Arrhenius plot for TmTK with thymidine and ATP. The  $v_{\max}$  values were measured by radioassay at the specified temperatures.

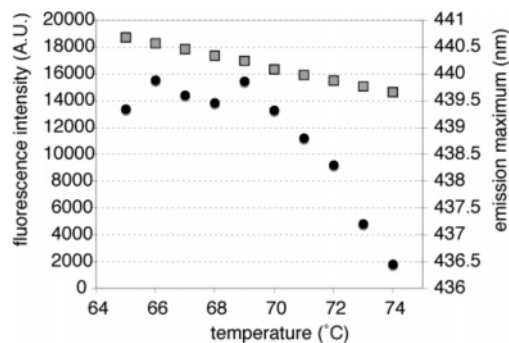
37 °C, yet does not explain TmTK's broadened substrate specificity compared to that of human TK1.

A more promising lead to explain the specificity change might be TmTK's strikingly similar catalytic rates for all tested nucleosides at 37 °C (Table 2). Mesophilic TK activity for the same substrates differs by up to 2 orders of magnitude.<sup>10</sup> The rate homogeneity has been rationalized by a common intermediate preceding the rate-limiting step in the reaction pathway.<sup>11,12</sup> While TmTK's direct phosphoryl-transfer mechanism makes a chemical intermediate implausible, the uniform  $k_{\text{cat}}$  values are consistent with a common structural intermediate, resulting from a substrate-independent conformational change as the rate-determining step at 37 °C.

Evidence supporting such a structural change was found upon analysis of enzyme reaction rates at different temperature via Arrhenius plot (Figure 1). The data revealed a discontinuity at 70 °C, indicating a change in the rate-limiting step. Above the transition temperature, the slope corresponds to an activation energy of ~4 kcal/mol, while below 70 °C, the energy barrier increases to 16 kcal/mol. A conformational change as the enzyme's slow-step at temperatures below 70 °C could explain the equal reaction rates.

Independent verification for a temperature-dependent conformational change in TmTK was obtained by fluorescence spectroscopy. The crystal structure of the TmTK homotetramer suggests a transition at the dimer II interface upon ATP binding, shifting the equilibrium from a closed, inactive state to a catalytically competent, open conformation.<sup>5,13</sup> Speculating that this transition could take place spontaneously at elevated temperature and correlates with our observed kinetic effect, we used mant-ADP, an ADP analogue with an environmentally sensitive fluorescent *N*-methylantraniloyl portion at the 2'- or 3'-position of the adenosine, to monitor conformational changes at the dimer II interface.

Our experiments detected a transition point in the fluorophore's emission wavelength at 70 °C (Figure 2). Monitoring the fluorescence intensity and  $\lambda_{\text{em}}$  of mant-ADP in the presence of T, we detected a biphasic pattern where  $\lambda_{\text{em}}$  showed minimal temperature dependence below 70 °C, yet blue shifted by 0.5–0.8 nm/°C above the transition temperature. While initial scans covered a temperature range from 37 to 90 °C, subsequent measurements in one-degree increments focused on a temperature range from 65 to 75 °C. As T/mant-ADP represents a mixed substrate/product complex, we repeated the experiments with TMP/mant-ADP and observed the same  $\lambda_{\text{em}}$  transition above 70 °C, although with a more moderate slope of 0.2 nm/°C (data not shown). Control experiments with mant-ADP in the absence of TmTK show no significant temperature dependence of  $\lambda_{\text{em}}$ . The observed blue shift indicates a repositioning of the fluorophore in a more hydrophobic environment above 70 °C, consistent with the expansion at the dimer II interface.<sup>5</sup>



**Figure 2.** Fluorescence spectroscopy to detect temperature-dependent structural changes in TmTK via mant-ADP. The fluorescence intensity (□) and maximum emission wavelength (●) were plotted against temperature.

In summary, our study of the TK from the *T. maritima* revealed a significant, temperature-dependent change in substrate specificity. While preferring thymidine to AZT at physiological conditions (82 °C), the enzyme shows substrate promiscuity at 37 °C. We have used kinetic and spectroscopic evidence to link this phenomenon to a conformational change in TmTK at 70 °C. Concurrent with structure data, we propose a two-state model for TmTK involving a thermodynamically preferred but inactive closed state and a catalytically competent open structure of the TmTK homotetramer. While in rapid equilibrium above 70 °C, the transition from closed to open state becomes the rate-limiting step of the reaction pathway at lower temperatures. Consequently, that leads to a normalization of the catalytic rates of phosphorylation and the observed substrate promiscuity of TmTK.

With respect to enzyme evolution and biocatalysis, future experiments will explore the generality of substrate promiscuity among enzymes from extremophiles and investigate the structural and functional consequences of changing protein dynamics in detail.

**Acknowledgment.** We acknowledge financial support in part by the NIH (GM69958) and by a grant to the Emory Center for AIDS Research (AI050409) from the NIH and by institutional funding from the Emory University HSC. We thank Dr. Raymond Schinazi for providing samples of the L-nucleosides and DOT.

**Supporting Information Available:** Full experimental details (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA0734391