Characterization of *Streptococcus pneumoniae* thymidylate kinase: steady-state kinetics of the forward reaction and isothermal titration calorimetry

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Thymidylate kinase (TMK) catalyses the phosphorylation of dTMP to form dTDP in both the *de novo* and salvage pathways of dTTP synthesis. The *tmk* gene from the bacterial pathogen *Streptococcus pneumoniae* was identified. The gene, encoding a 212-amino-acid polypeptide (23352 Da), was cloned and overexpressed in *Escherichia coli* with an N-terminal hexahistidine tag. The enzyme was purified to homogeneity, and characterized in the forward reaction. The pH profile of TMK indicates that its activity is optimal at pH 8.5. The substrate specificity of the enzyme was examined; it was found that not only ATP, but also dATP and to a lesser extent CTP, could act as phosphate donors, and dTMP and dUMP could serve as phosphate acceptors.

Furthermore, AZT-MP (3'-azido-3'-deoxythymidine 5'-monophosphate) was shown not to be a substrate for *S. pneumoniae* TMK. Steady-state kinetics and inhibition studies with adenosine 5'-[β -thio]diphosphate and dTDP in addition to isothermal titration calorimetry were performed. The data showed that binding follows an ordered pathway, in which ATP binds first with a K_m of $235 \pm 46 \mu\text{M}$ and a K_d of $116 \pm 3 \mu\text{M}$, and dTMP binds secondly with a K_m of $66 \pm 12 \mu\text{M}$ and a K_d of $53 \pm 2 \mu\text{M}$.

Key words: AZT-MP, dissociation constants, dTMP kinase, kinetic mechanisms, nucleotide specificity.

INTRODUCTION

Streptococcus pneumoniae is one of the leading causes of septicæmia, meningitis and lower-respiratory-tract infections in humans. Despite rapid and aggressive antibiotic therapy, pneumococcal infection can lead to a variety of debilitating complications, including hearing loss. A better understanding of the cellular biochemistry of the pneumococci is essential for the development of novel anti-microbial therapeutics.

Thymidylate kinase (dTMP kinase or TMK; EC 2.7.4.9) catalyses the terminal phosphoryl group transfer from ATP to dTMP in the biosynthesis of the pyrimidine deoxyribonucleotide, dTTP. The cascade of enzymes that activate nucleosides to nucleoside triphosphates and deoxynucleoside triphosphates (NTPs and dNTPs respectively) is of interest in drug discovery, in that these molecules are vital precursors in the synthesis of DNA, RNA and other cellular macromolecules (peptidoglycan in bacteria). TMK is no exception, and has been extensively studied both as an anti-viral target and for its potential role in cancer therapy. Variability in the active site residues and catalytic properties of TMK enzymes from various sources (viral, eukaryotic and bacterial) has opened the possibility of designing specific and selective inhibitors. The essential nature of TMK for bacterial growth ([1]; C. Petit, unpublished work), and the fact that the protein is found in both Gram-negative and Gram-positive human bacterial pathogens, render this enzyme an attractive target for the development of novel, broad-spectrum anti-bacterial agents.

TMK enzymes are globular dimeric proteins with a similar fold to that found in other nucleoside monophosphate kinases [2], with three loops crucial for enzyme activity: the phosphate-binding motif at the N-terminus (P-loop), the nucleoside-monophosphate-binding domain, and the region which covers part of the P-loop upon substrate binding (LID domain). Variability in

phosphorylation of the anti-viral pro-drug 3'-azido-3'-deoxythymidine 5'-monophosphate (AZT-MP) was first identified when comparing the activity of the bacterial and eukaryotic TMK enzymes [2–5]. Kinetic measurements with the enzyme from an *Escherichia coli* source (TMK_{coli}) showed that the maximum rate of AZT-MP phosphorylation is reduced only by a factor of 2 in comparison with dTMP, whereas this reduction is 200-fold for TMK_{yeast} and TMK_{human} [3,6,7]. Crystal structures of TMK_{yeast} complexed with dTMP [5], AZT-MP [5] and P¹-(5'-adenosyl)-P⁵-(5'-thymidyl)pentaphosphate (TP₅A) [8], and structures of TMK_{coli} complexed with TP₅A and with P¹-(5'-adenosyl)-P⁵-(5'-[3'-azido-3'-deoxythymidine]) pentaphosphate ('AZT-P₅A') [6] were solved. Analysis of these structures indicated that the reduction in the maximum rate of AZT-MP phosphorylation in eukaryotic TMKs is the result of a transition-state-stabilizing arginine residue located in the P-loop being shifted out of place by the movement of the adjacent invariant aspartate residue due to steric interactions with the amide group of AZT-MP. In contrast, this essential arginine residue is located in the LID region, not the P-loop, in TMK_{coli}. Consequently, the P-loop does not shift when binding AZT-MP and phosphorylation can occur efficiently. These reports prompted Chenal-Francis et al. [7] to investigate further bacterial TMK enzymes. Characterization of the *Yersinia pestis* enzyme (TMK_{pestis}) highlighted a striking difference in terms of its ability to phosphorylate AZT-MP compared with TMK_{coli}. The V_{max} obtained with AZT-MP as substrate was only 1% that obtained with dTMP for TMK_{pestis}, whereas only a 2-fold difference in V_{max} was observed between AZT-MP and dTMP for TMK_{coli}. Even though the two bacterial enzymes share higher sequence homology than they do with their eukaryotic counterparts, the *Y. pestis* enzyme behaves more similarly to the yeast and human enzymes than to the *E. coli* enzyme. This variation in V_{max} was demonstrated further with *Haemophilus influenzae* and *Salmonella typhi*: the maximum

Abbreviations used: TMK, thymidylate kinase; AZT-MP, 3'-azido-3'-deoxythymidine 5'-monophosphate; ADP[β -S], adenosine 5'-[β -thio]diphosphate; TMK_{coli}, TMK from an *Escherichia coli* source, etc.; ITC, isothermal titration calorimetry; ATP[S], adenosine [γ -thio]triphosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid.

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rate of phosphorylation of AZT-MP in the former was more similar to that of TMK_{pestis}, while the maximum rate in the latter was more similar to that of TMK_{coli} [7]. When comparing the protein sequences of TMK_{pestis} and TMK_{coli}, three non-conserved residues within the active site were identified, which might account for the variability in the maximum phosphorylation rate for AZT-MP (Lys⁵⁵, Leu⁷² and Ser¹⁰³ in TMK_{pestis} replaced by Leu⁵⁵, Phe⁷⁴ and Thr¹⁰⁵ in TMK_{coli}) [7]. Interestingly, the authors also reported that TMK from another Gram-positive human pathogen, *Mycobacterium tuberculosis*, does not use AZT-MP as a substrate. A preliminary X-ray-crystallographic analysis of TMK_{tuberculosis} complexed with dTMP was reported recently, which should clarify further its substrate specificity [1].

The kinetic mechanism of mouse TMK and those of the homologous enzymes guanylate kinase and adenylate kinase have been shown to be random Bi Bi in nature, i.e. in which the nucleotides bind independently of each other [9–11]. Recent studies on the mechanism of the human enzyme implied that it too would function with random binding of the substrates [2]. However, no data are available on the catalytic mechanism of their bacterial counterparts.

In an effort to investigate the kinetic mechanism of TMK from the human pathogen *S. pneumoniae*, the gene was cloned and expressed in *E. coli* and the protein was subsequently purified. The enzyme was characterized for its substrate specificity. Results obtained from initial-velocity experiments and by isothermal titration calorimetry (ITC) suggest that the mechanism is ordered, with ATP binding first and dTMP binding secondly. We show that the streptococcal enzyme does not utilize AZT-MP as a substrate, as is the case for other bacterial enzymes, and discuss this difference.

EXPERIMENTAL

Materials

All chemicals were of analytical grade, and were used without further purification. The nucleotides dTMP, dTDP, adenosine 5'-[β -thio]diphosphate (ADP[β -S]) and 5'-[γ -thio]triphosphate ATP[S], the coupling enzymes pyruvate kinase and lactate dehydrogenase, phosphoenolpyruvate, NADH and the buffer reagents Mes, Tris, Hepes, and Ches [2-(*N*-cyclohexylamino) ethanesulphonic acid] were from Sigma (St Louis, MO, U.S.A.). ATP was from Roche Applied Science (Indianapolis, IN, U.S.A.).

Cloning, expression and purification

The *S. pneumoniae tmk* gene was PCR-amplified from genomic DNA using the following primers: 5'-GAGTTACAT **ATGTCA**-AAAGGATTTT**AGTCTCTC**-3' and 5'-GTTGATGAATTC-TTATTTGGCCAAGCCC-3'. These primers were engineered to create an *Nde*I site at the N-terminus of the gene, and an *Eco*RI site at the C-terminus (sequences of the restriction enzyme sites are shown in bold, and the start and stop codons are underlined). The gene was isolated and cloned into pET28a(+) as an *Nde*I-*Eco*RI fragment to encode an N-terminally hexahistidine-tagged protein with a thrombin cleavage site after the tag. The construct was expressed in *E. coli* strain BL21(DE3), and induced with 1 mM isopropyl β -D-thiogalactoside ('IPTG') at 37 °C for 3 h in Luria-Bertani medium containing 50 μ g $\cdot$ ml⁻¹ kanamycin. The TMK protein was expressed at a level of approx. 25% of the total protein (of which about 50% was soluble), as judged by SDS/PAGE. The protein was then purified to >95% homogeneity by Ni²⁺-chelate chromatography, using a HiTrap chelat-

ing column and the conditions recommended by the manufacturer (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Enzyme assay

All the reactions were performed in 96-well plates (Labsystems, Helsinki, Finland) in an assay volume of 100 μ l. The TMK activity was measured at 25 °C by coupling the formation of ADP with the reaction catalysed by pyruvate kinase (4 units) and lactate dehydrogenase (4 units) in the presence of 50 mM KCl, 1 mM phosphoenolpyruvate and 0.1 mM NADH. The reaction was initiated by the addition of 20 nM TMK, except when stated otherwise. The molar ratio of MgCl₂:ATP was kept constant at 2. Oxidation of NADH was monitored by fluorescence (λ_{ex} = 355 nm; λ_{em} = 460 nm) using a BMG PolarStar plate reader (BMG Labtechnologies Inc., Durham, NC, U.S.A.). The activities are expressed as arbitrary units $\cdot$ s⁻¹. All data fitting was performed with non-linear least-squares regression, using the commercial software package Graphit 4.0 (Erithacus Software, Horley, Surrey, U.K.).

ITC

ITC experiments were performed using a VP-ITC titration microcalorimeter (Microcal Inc., Northampton, MA, U.S.A.). The reference cell was filled with water. The TMK protein was dialysed overnight in 50 mM Hepes buffer, pH 8.5, containing 5 mM MgCl₂. Substrate solutions were prepared in the final dialysis buffer. Solutions of the TMK protein were filled in the sample cell (1.4 ml volume) and titrated with ATP[S] or dTMP solutions. ITC experiments were routinely performed at 25 °C. Raw data were collected and integrated using the Microcal Origin software supplied with the instrument. The data were fitted to a single-site binding model by a non-linear regression analysis to yield binding constants (K_b), enthalpies of binding (ΔH) and the stoichiometry of binding.

Other methods

The N-terminal amino acid sequence of the purified enzyme was deduced by automated Edman degradation with a Hewlett-Packard (Boise, ID, U.S.A.) model G1000A sequencer. The molecular mass of the enzyme was determined by matrix-assisted laser-desorption ionization-time-of-flight MS using a PerSeptive Biosystems Voyager RP Biospectrometer (Framingham, MA, U.S.A.). Protein concentration was determined by amino acid hydrolysis.

RESULTS

S. pneumoniae histidine-tagged TMK was overexpressed in *E. coli* and purified to homogeneity by Ni²⁺-chelate chromatography (results not shown). The effect of pH on enzyme activity was examined. The buffers used were Ches, Tris and Mes, all at a concentration of 50 mM. The ionic strength was held constant at 50 mM by adding various concentrations of NaCl (see <http://www.bi.umist.ac.uk/users/mjfrbn/Buffers/Makebuf.asp>). TMK was found to have an optimum activity between pH 8 and pH 9 (Figure 1). The effect of NaCl was examined further in 50 mM Tris/HCl, pH 8.5. No significant change was observed when the NaCl concentration was varied from 0–50 mM (results not shown), and therefore the remaining experiments were

Figure 1 Effect of pH on *S. pneumoniae* TMK activity

The assays were performed as described in the Experimental section using the following buffers: 50 mM Mes for pH measurements between pH 5.6 and 6.7, 50 mM Tris/HCl for pH 7.1–9, and 50 mM Ches for pH 9.2–9.9. Velocities are expressed in arbitrary units (AU) · s⁻¹. Each point represents the average of experiments performed in duplicate.

Table 1 Specificity of *S. pneumoniae* TMK for nucleotides

Experiments were performed at 25 °C and pH 8.5. Activities are expressed as percentages in comparison with a control using ATP and dTMP as substrates. The concentration of each nucleotide added to the reaction was 2 mM. In the case of AZT-MP, activity was tested with up to a 100-fold greater enzyme concentration as compared with the amount used when testing the other phosphate acceptors.

Nucleotides (phosphate donors)	Relative activity (%)	Nucleotides (phosphate acceptors)	Relative activity (%)
ATP	100	dTMP	100
dATP	80	dUMP	15
CTP	20	UMP	0
dCTP	0	AMP, dAMP	0
TTP, dTTP	0	CMP, dCMP	0
GTP, dGTP	0	GMP, dGMP	0
UTP	0	AZT-MP	0

performed in 50 mM Tris at pH 8.5, in the absence of NaCl. To determine substrate specificity, various NTPs or dNTPs were tested as phosphate donors in combination with NMPs or dNMPs as phosphate acceptors. Not only ATP, but also dATP (reaction velocity 80% of that of ATP) and, to a lesser extent, CTP (reaction velocity 20% of that of ATP) could act as phosphate donors (Table 1). Of the NMPs tested, only dTMP and, to a lesser extent, dUMP (reaction velocity 15% of that of dTMP) could act as phosphate acceptors for *S. pneumoniae* TMK (Table 1). Interestingly, no activity was detected when using AZT-MP as a phosphate acceptor, even when the amount of enzyme was increased 100-fold (2 μM) as compared with the amount used for the other nucleotides (20 nM; Table 1).

Initial velocity analysis

The kinetics of the forward reaction (formation of ADP and dTDP) was measured by coupling the reaction with those of pyruvate kinase and lactate dehydrogenase [12]. Enzyme assays were performed in a 5 × 5 array with various concentrations of

Figure 2 Double-reciprocal plots of substrate concentration against the initial velocities

The concentration of ATP was varied from 100–800 μM, while the concentration of dTMP was held constant at 25 (●), 50 (○), 75 (■), 100 (□) or 150 (×) μM. Initial velocities were fitted to eqn (1). Velocities are expressed in arbitrary units · s⁻¹. Each point represents the average of experiments performed in duplicate.

ATP (100–800 μM) and dTMP (25–150 μM). Initial velocities were fitted to eqn (1):

$$v = \frac{V_{\max} AB}{K_a K_b + K_b A + K_a B + AB} \quad (1)$$

where A and B represent the concentration of ATP and dTMP respectively. The double-reciprocal plots of the kinetic data are linear, and they intersect, which is indicative of a ternary mechanism (Figure 2). A standard curve with NADH concentrations ranging from 0–100 μM was performed. The velocities obtained in arbitrary units of fluorescence · s⁻¹ were then converted into units of μM NADH oxidized · s⁻¹ to calculate k_{cat} , and the second-rate constant k_{cat}/K_m . The kinetic parameters are summarized in Table 2.

Product inhibition studies

Since the reaction product ADP is a substrate for the coupling enzyme pyruvate kinase used in the assay, product inhibition experiments were performed using the non-hydrolysable product analogue ADP[β-S]. The concentrations tested were 0, 50, 100 and 200 μM. The data fitted best to a competitive-inhibition model against ATP (eqn 2), and a non-competitive model against dTMP (eqn 3; Figures 3A and 3B). Similarly, inhibition patterns with dTDP were obtained at various concentrations. Data were fitted to a non-competitive model against ATP (eqn 3) and a competitive-inhibition model against dTMP (eqn 2; Figures 4A and 4B). The inhibition constants are summarized in Table 3.

$$v = \frac{V_{\max} A}{K_a(1 + I/K_i) + A} \quad (2)$$

$$v = \frac{V_{\max} A}{(K_a + A)(1 + I/K_i)} \quad (3)$$

ITC experiments

Since Mg²⁺ is required for the phosphorylation reaction catalysed by *S. pneumoniae* TMK, and since it could influence the

Table 2 Steady-state kinetic parameters of the forward reaction of *S. pneumoniae* dTMP kinase

The initial velocity data were fitted to eqn (1). Experiments were performed at 25 °C and pH 8.5.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
ATP	235 ± 46	8.9 ± 0.9	3.8×10^4
dTMP	66 ± 12	8.9 ± 0.9	1.3×10^5

equilibrium binding parameters by either direct participation in the bonding network of the binding pocket or by indirect structural effects, all measurements were performed in the presence of 5 mM MgCl_2 . To prevent enzymic turnover in the ITC experiments, the non-hydrolysable substrate analogue ATP[S] was used instead of ATP. *S. pneumoniae* TMK was titrated with either ATP[S] or dTMP, and Figure 5 shows representative

Table 3 Dead-end inhibition pattern for *S. pneumoniae* TMK. The initial velocity data were fitted to eqns (2) and (3)

Experiments were performed at 25 °C and pH 8.5. C, competitive; NC, non-competitive.

Inhibitors	Variable substrate	Inhibition pattern	Inhibition constants (K_{is} ; μM)
ADP[β -S]	ATP	C	47 ± 3
	dTMP	NC	415 ± 25
dTMP	ATP	NC	1550 ± 73
	dTMP	C	160 ± 8

titrations. The titration with ATP[S] was characterized by a significant exothermic heat effect, with a ΔH of $-10.1 \text{ kcal} \cdot \text{mol}^{-1}$ ($1 \text{ cal} \equiv 4.184 \text{ J}$). The binding constant K_B was determined to be $8605 \pm 222 \text{ M}^{-1}$ (means $\pm$ S.E.M., provided by the ITC software when fitting data), which corresponds to a K_d of $116 \pm 3 \mu\text{M}$. In contrast, titration with dTMP unveiled a very different kinetic behaviour: no heat effect typical of a binding reaction was

Figure 3 Double-reciprocal plots of ATP and dTMP concentrations against the initial velocity for TMK activity in presence of ADP[β -S]

(A) The concentration of dTMP was held constant at $200 \mu\text{M}$ and the concentration of ATP was varied. The concentrations of ADP[β -S] were 0 ($\bullet$), 50 ($\circ$), 100 ($\blacksquare$) and $200 (\square) \mu\text{M}$. (B) The concentration of ATP was held constant at $300 \mu\text{M}$ and the concentration of dTMP varied. The concentrations of ADP[β -S] were 0 ($\bullet$), 100 ($\circ$), 200 ($\blacksquare$) and $400 (\square) \mu\text{M}$. Velocities are expressed in arbitrary units $\cdot \text{s}^{-1}$. Each point represents the average of experiments performed in duplicate.

Figure 4 Double-reciprocal plots of ATP and dTMP concentrations against the initial velocity for TMK activity in presence of dTDP

(A) The concentration of dTMP was held constant at $200 \mu\text{M}$ and the concentration of ATP was varied. The concentrations of dTDP were 0 ($\bullet$), 600 ($\circ$), 1200 ($\blacksquare$) and $1800 (\square) \mu\text{M}$. (B) The concentration of ATP was held constant at $300 \mu\text{M}$ and the concentration of dTMP was varied. The concentrations of dTDP were 0 ($\bullet$), 100 ($\circ$), 200 ($\blacksquare$) and $350 (\square) \mu\text{M}$. Velocities are expressed in arbitrary units $\cdot \text{s}^{-1}$. Each point represents the average of experiments performed in duplicate.

Figure 5 Representative isothermal titration calorimetry measurements

Titration were performed at 25 °C in 50 mM Hepes, pH 8.5, containing 5 mM MgCl_2 . Of the TMK enzyme, 62 μM was titrated with 2.4 mM ATP[S] (A) or 3 mM dTMP (B). TMK enzyme (62 μM) in the presence of 2 mM ATP[S] was titrated with 3 mM dTMP (C). The upper panels are untreated data shown as differential power signals (expressed in $\mu\text{cal} \cdot \text{s}^{-1}$). The lower panels are binding isotherms obtained by integration of the raw data (expressed in $\text{kcal} \cdot \text{mol}^{-1}$ of injectant).

observed, indicating that dTMP does not bind the free TMK enzyme. Furthermore, no heat change was detected when titrating the free enzyme to dTDP (results not shown). These results suggest that only ATP[S] can bind to the free form of TMK. To confirm an ordered binding mechanism in which $\text{TMK} \cdot \text{ATP}$ precedes the formation of $\text{TMK} \cdot \text{ATP} \cdot \text{dTMP}$, the pre-formed $\text{TMK} \cdot \text{ATP[S]}$ binary complex (62 μM TMK and 2 mM ATP[S]) was titrated with dTMP. This reaction was slightly endothermic, with a ΔH of 1.3 $\text{kcal} \cdot \text{mol}^{-1}$, and yielded a value for K_B of $1.89 \times 10^4 \pm 750 \text{ M}^{-1}$, which corresponds to a K_d of $53 \pm 2 \mu\text{M}$. The stoichiometry of ATP[S] binding to TMK and of dTMP binding to the $\text{TMK} \cdot \text{ATP[S]}$ binary complex was 1.

DISCUSSION

TMK has an essential role in both the *de novo* and salvage pathways of dTTP synthesis, with dTTP serving as a precursor in the synthesis of DNA. The role of TMK renders it an attractive target for the development of anti-microbial agents. The differences between eukaryotic and prokaryotic TMK proteins within the active site, taken together with the lack of mechanistic data in the literature, prompted us to undertake the characterization of the kinetic mechanism of TMK from the human pathogen *S. pneumoniae*.

The mechanism of the forward reaction was explored using both steady-state kinetics and ITC. Double-reciprocal plots of the initial-velocity data disclosed intersecting lines. These results are characteristic of a sequential mechanism. Both substrates behaved as Michaelis–Menten substrates over the range of concentrations studied. The K_m values of ATP and dTMP were $235 \pm 46 \mu\text{M}$ and $66 \pm 12 \mu\text{M}$ respectively; the k_{cat} was calculated to be $8.9 \pm 0.9 \text{ s}^{-1}$. These values are in the same order of magnitude as that reported for the TMK enzymes of *E. coli* and *Y. pestis* [7], embryonic chick liver [13], and cells of human chronic myelocytic leukaemia [14] or HSV-1- and HSV-2-infected cells [15]. Using ADP[β -S] as an analogue of ADP and dTDP, it was not possible to distinguish with certainty between a random and an obligatory order of addition. From the inhibition studies, the mode of inhibition with ADP[β -S] was competitive against ATP and non-competitive against dTMP, and with dTDP the inhibition was non-competitive against ATP and competitive against dTMP. However, we showed by ITC that only ATP[S] could bind to the free TMK, unlike dTMP or dTDP. Binding of dTMP was observed only when the complex $\text{TMK} \cdot \text{ATP[S]}$ was pre-formed. These results support an ordered Bi Bi mechanism, and would differ from those obtained for mouse TMK [9] or that suggested by the structural results obtained with the human enzyme [2]. No kinetic mechanism has been proposed as yet for the bacterial TMKs, and it is possible to envisage major differences between bacterial and non-bacterial enzymes.

The substrate specificity was evaluated. Of the NMPs tested, dTMP was the best phosphate acceptor. The results for the nucleoside triphosphates were very similar to those reported in *E. coli* and *Y. pestis*, with ATP and dATP being effective as phosphate donors, and CTP being moderately effective as a phosphate donor [7]. However, an unexpected difference between *S. pneumoniae* TMK and the enzyme from other sources (*E. coli*, *Y. pestis*, *S. typhi*, *H. influenzae*, *Homo sapiens*, *S. cerevisiae*) is that AZT-MP is not used as substrate by the former, but was reported to be a substrate for the latter enzymes [3,6,7]. No activity could be detected with AZT-MP, even in the presence of a concentration of *S. pneumoniae* TMK that was 100-fold higher than that used with dTMP. Similarly to *S. pneumoniae* TMK, the enzyme from the Gram-positive human pathogen *M. tuberculosis*, a bacterium insensitive to AZT, does not use AZT-MP as a

	P-loop	helix 2	helix 3	strand 4	LID
<i>S. pneumo</i>	³⁰ FLVSL ³¹ EGPEGAGKTSV ³²	⁴⁵ LIG ⁴⁶ EKIREVIL ⁴⁷ D ⁴⁸	⁶⁸ LMLYA ⁶⁹ ARVQLV ⁷⁰	⁹³ VVGDRHDLSSQ ¹⁰³	¹⁴³ GLAR ¹⁴⁴ IaANSd ¹⁴⁵ re----VNRLDL ¹⁶⁰
<i>KTHY_YERPE</i>	⁴³ KFIVIEGLEGAGKTTT ⁴⁹	⁴³ PLAEKLRDLIKQ ⁵⁶	⁶⁸ LMLYA ⁶⁹ ARIQLV ⁷⁰	⁹⁴ VVGDRHDMSSQ ¹⁰⁴	¹⁴⁴ GLARARARGe-----LDRIEQ ¹⁵³
<i>H. influ</i>	⁴³ KFIVIEGLEGAGKSSA ⁴⁹	⁴³ PLAEKLRHLIKH ⁵⁶	⁶⁸ LMFYA ⁶⁹ ARVQLV ⁷⁰	⁹³ VIGDRHDLSTQ ¹⁰³	¹⁴³ GLKRARGRGe-----LDRIEQ ¹⁵⁸
<i>KTHY_ECOLI</i>	⁴³ KYIVIEGLEGAGKTTA ⁴⁹	⁴⁵ QLAEKLRSLLLD ⁵⁶	⁷¹ LMFYA ⁶⁹ ARVQLV ⁷⁰	⁹⁶ VIGDRHDLSTQ ¹⁰⁶	¹⁴⁶ GLKRARARGe-----LDRIEQ ¹⁶¹
<i>S. typhi</i>	⁴³ NYIVIEGLEGAGKTTA ⁴⁹	⁴⁵ QLAEKLRSLVL ⁵⁶ D ⁵⁷	⁷¹ LLYIASRRQHL ⁸²	⁹⁶ VIMDRFIDSSV ¹⁰⁶	¹⁴⁶ GLARARARGe-----LDRIEQ ¹⁶¹
<i>M. tuber</i>	⁴³ MLIAIEGVDGAGKRTL ⁴⁹	⁴³ VAADIAAEALHG ⁵⁴	⁷¹ TLFALDRAGAV ⁷⁸	⁹¹ VILDRYVASNA ¹⁰¹	¹⁴⁶ AGERSrGRAqr ¹⁴⁷ dpgrardNYER ¹⁶⁷
<i>S. aureus</i>	⁴³ AFITFEGPEGS ⁴⁴ GKTTV ⁴⁹	⁴³ PTGEEIRKIVLE ⁵⁶	⁶⁸ MLFAASRREHL ⁷⁷	⁹¹ VLC ⁹² DRYIDSS ⁹³ L ¹⁰¹	¹⁴³ GRERI ¹⁴⁴ knsg ¹⁴⁵ rd-----QNRLDQ ¹⁵⁷
<i>KTHY_PSEAE</i>	⁴³ LFVTLEGPEGAGKSTN ⁴⁹	⁴³ PLAERIRELLLA ⁵⁵	⁶⁸ LLMFAARAQHL ⁷⁸	⁹² VLC ⁹³ DRFTDATY ¹⁰²	¹⁴³ GLARAAaARGr-----LDRFEQ ¹⁵⁷
<i>KTHY_THEMA</i>	⁴³ MFITFEGIDGSGKSTQ ⁴⁹	⁴³ ETGEKIRKILLE ⁵⁵	⁶⁸ LFLFLASRNLL ⁷¹	⁹³ VLLDRYT ⁹⁴ DSSV ⁹⁵	¹³⁹ ALKRKge-----LNRF ¹⁴⁰ EK ¹⁴⁷
<i>KTHY_CAUCR</i>	⁴³ KFIVIEGLEGAGKTTT ⁴⁹	⁴³ PLAEKLRDLIKQ ⁵⁶	⁶⁸ LMLYA ⁶⁹ ARVQLV ⁷⁰	⁹⁴ VVGDRHDLSSQ ¹⁰⁴	¹⁴⁴ GLARARARGe-----LDRIEQ ¹⁵⁹
<i>KTHY_UREPA</i>	⁴³ LFIVFEGIDGAGKTSI ⁴⁹	⁴³ NAAEMIREFFFLK ⁷²	⁸³ YLYASSRAEHV ⁹⁴	¹⁰⁸ VISDRFVHSSY ¹¹⁸	¹⁵⁸ ALNRMknz ¹⁵⁹ fdn-----TNAFDS ¹⁷⁴
<i>KTHY_BACSU</i>	⁴³ LFITFEGPEGAGKTTV ⁴⁹	⁴³ DIAEQIREVILN ⁴⁹	⁶⁷ LLYAAARRQHL ⁷⁸	⁹² VLC ⁹³ DRFIDSS ⁹⁴ L ¹⁰²	¹⁴² GLKRIYANGsre-----KNRLDL ¹⁵⁹
<i>1E2DA</i>	³⁵ ALIVLEGVDRAGKSTQ ³⁸	⁵¹ EIGKLLSSYLQK ⁶²	⁷² LLFSANRWEQV ⁸³	⁹⁶ LVVDYAFSGV ¹⁰⁶	¹⁴³ AAKRG-AFG-----HERYEN ¹⁵⁶
<i>2tmkA</i>	⁴¹ KLILIEGLDRTGKTTQ ⁴⁹	⁴³ RIGGLINEYLT ⁴⁵ D ⁴⁶	⁶⁸ LLFSANRWEIV ⁷⁷	⁹⁰ IVMDRYVYSGV ¹⁰⁰	¹⁴⁶ NAEKS-GFG-----DERYET ¹⁵³
<i>KTHY_CAEEL</i>	⁴¹ LLIVFEGLD ⁴² RS ⁴³ GKSTQ ⁴⁶	⁵⁹ SIGKLIDQYL ⁶⁹ RK ⁷⁰	⁶⁸ LLFSADRF ⁶⁹ SKN ⁷¹	¹⁰⁶ VIC ¹⁰⁷ DRYCYSGV ¹¹⁴	¹⁵⁶ AAQRG-GFG-----EERLET ¹⁶³

Figure 6 Multiple sequence alignment of dTMP kinases

Only the P-loop, the nucleotide-binding, the phosphate-binding and the LID regions are represented. Species featured are *S. pneumoniae* (*S. pneumo*), *Y. pestis* (*KTHY_YERPE*), *H. influenzae* (*H.influ*), *E. coli* (*KTHY_ECOLI*), *S. typhi* (*S typhi*), *M. tuberculosis* (*M tuber*), *S. aureus* (*S aureus*), *Pseudomonas aeruginosa* (*KTHY_PSEAE*), *Thermotoga maritima* (*KTHY_THEMA*), *Caulobacter crescentus* (*KTHY_CAUCR*), *U. parvum* (*KTHY_UREPA*), *B. subtilis* (*KTHY_BACSU*), *H. sapiens* (*1E2DA*), *S. cerevisiae* (*2tmkA*) and *C. elegans* (*KTHY_CAEEL*). Numbers adjacent to the residues refer to the position of the first and last amino acids of each region. Residues that are thought to be involved in phosphorylation are underlined: those that are conserved with *E. coli* are singly underlined, and those that are variable are doubly underlined. Lower-case residues indicate non-alignable residues across all species in the multiple sequence alignment.

substrate [7]. The same authors observed that *S. typhi* TMK phosphorylates AZT-MP at a maximum rate comparable with that observed with TMK_{coli} (2–5-fold reduction in $V_{\max}$), whereas TMK_{influenzae} is more similar to TMK_{pestis} (100-fold reduction in $V_{\max}$) [7].

To gain a further insight into the substrate specificity of the TMK enzymes, we compared the primary protein structures of twelve bacterial and three eukaryotic enzymes (Figure 6). From previous studies of structure and sequence comparisons, it was noted that the variation in maximum phosphorylation rates of AZT-MP between *E. coli* and eukaryotic TMK enzymes was most likely due to the position of an essential arginine, which is located in the P-loop of eukaryotic TMK enzymes and in the LID region in bacterial TMK enzymes. The rationale explaining the variation observed amongst the bacterial species *E. coli*/*S. typhi* and *Y. pestis*/*H. influenzae* was a little harder to identify. From structure and sequence analyses, it was hypothesized that the variation in $V_{\max}$ of AZT-MP phosphorylation is due to the variability in a few residues within the active site [7]. Further analysis of the primary structures indicates that the main mutation responsible for the variability in bacteria is the leucine → lysine substitution (residue 55 in *E. coli*, *Y. pestis*, *S. typhi*, and *H. influenzae*), since it is the only mutation consistent with the activity data obtained with AZT-MP. This residue is conserved between *S. pneumoniae* and *E. coli* (Figure 6). Therefore one would initially expect the $V_{\max}$ to be similar. The complete lack of phosphorylation of AZT-MP in *S. pneumoniae* must therefore be due to some other mutation(s). The only other variability between *E. coli* and *S. pneumoniae* around the phosphate-binding site is in the LID region, with the *S. pneumoniae* LID region having two extra residues. In fact, the arginine residue that is needed to stabilize the transition state is not in the same location within the LID. This difference holds true for the *Mycobacterium* enzyme, which has an even larger LID region. Without crystal-

structure data on *S. pneumoniae*, we can only speculate that the larger amide group on AZT-MP forces the arginine out of position and therefore phosphoryl transfer cannot be achieved. If this is true, we should expect similar results for *Staphylococcus aureus*, *Bacillus subtilis* and *Ureaplasma parvum* (Figure 6).

In conclusion, kinetic parameters have been determined for the forward reaction catalysed by *S. pneumoniae* TMK. TMK catalyses the phosphoryl transfer via a sequential mechanism. The data presented here support an ordered mechanism. Formation of a binary complex of ATP with *S. pneumoniae* TMK is a stringent prerequisite for dTMP binding. This implies that ATP binding in fact generates the dTMP-binding site. In this case, one would expect the enzyme to undergo considerable conformation rearrangements. The details of such rearrangements must await a firm structural foundation. Work is currently in progress to solve the crystal structure of the apo form of *S. pneumoniae* TMK, as well as the forms of the enzyme in complex with substrate nucleotides and substrate analogues. The crystal structures will also help identify the segments lying outside the main nucleotide-binding and catalytic regions, which are responsible for the lack of AZT-MP phosphorylation in *S. pneumoniae*.

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