Bacterial Enzymes in Thymidylate Synthesis

Molecular Characterization of Thymidine Kinase and Thymidylate Kinase in *Ureaplasma urealyticum* and *Bacillus anthracis*; Implications for antibacterial therapy

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Abstract

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Deoxyribonucletides (dNTPs) are synthesized via the *de novo* and salvage pathways. The *de novo* synthesis involves initial synthesis of ribonucleotides whereas the salvage pathway uses deoxyribonucleosides. Deoxynucleoside kinases (dNKs) initiate the salvage pathway and this step is often considered as rate limiting. The two pathways eventually coincide to one where nucleoside monophosphate kinases are the shared enzymes.

In this thesis two thymidine kinases (TKs) and a thymidylate kinase (TMPK) are cloned, expressed and characterized and they originate from *Ureaplasma urealyticum* (*Uu*) and *Bacillus anthracis* (*Ba*). *Uu* causes urethritis and is associated with complications in pregnancy, e.g. premature births, infertility, spontaneous abortions and chronic lung disease in the infants. *Ba* causes anthrax; cutaneous, inhalational and gastrointestinal, where the two latter have a high mortality rate. *Ba* is also considered as a potent bioweapon because of its spore forming ability.

TK from *Uu* (*Uu*-TK) was strictly pyrimidine specific and used all nucleoside triphosphates as phosphate donors, except dTTP that was a feedback inhibitor. Further studies discovered that analogs with halogen substitutions at the 5-position gave the highest activity. Analogs with modifications at the N3- or 3'-position showed good to moderate activities while 2'-substituents were not substrates. A fluorine substitution was tolerated in the 2'-arabinosyl position. These results correlated well with the active site structure of *Uu*-TK. The *Uu*-TK structure contained a unique domain, the lasso domain with a structural zinc ion, and belonged structurally to another enzyme family than the other dNKs.

TK and TMPK from *Ba* (*Ba*-TK and *Ba*-TMPK) were strictly pyrimidine and thymidylate specific, respectively. *Ba*-TK used all nucleoside triphosphates as phosphate donors, except dTTP, and phosphorylated several nucleoside analogs. The analog activities of *Ba*-TK were similar to that of *Uu*-TK. *Ba*-TMPK used ATP and dATP as phosphate donors and a number of analogs as substrates. FMAUMP (1-(2-deoxy-2-fluoro-D-arabinofuranosyl)-5-methyluracil-5'-monophosphate) was the best substrate and its nucleoside form was a potent inhibitor.

Enzymes in thymidylate synthesis are potential targets for antibacterial therapy and the studies conducted in this thesis have discovered several potential leads, which will contribute to future design of antibiotics.

Keywords: thymidine kinase, thymidylate kinase, *Ureaplasma*, *Bacillus anthracis*, anthrax, nucleoside analog, *Uu*-TK structure

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To my family

Contents

Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Carnrot, C.*, Wehelie, R.*, Eriksson, S., Bölske, G., Wang, L. (2003) Molecular characterization of thymidine kinase from *Ureaplasma urealyticum*: nucleoside analogues as potent inhibitors of *mycoplasma* growth. *Mol Microbiol*. 50: 771-780. (*Equal contribution)

II. Welin, M., Kosinska, U., Mikkelsen, N-E., Carnrot, C., Zhu, C., Wang, L., Eriksson, S., Munch-Petersen, B., Eklund, H. (2004) Structures of thymidine kinase 1 of human and mycoplasmic origin. *Proc Natl Acad Sci U S A*. 101: 17970-17975.

III. Kosinska, U.*, Carnrot, C.*, Eriksson, S., Wang, L., Eklund, H. (2005) Structure of the substrate complex of thymidine kinase from *Ureaplasma urealyticum* and investigations of possible drug targets for the enzyme. *FEBS J*. 272: 6365-6372. (*Equal contribution)

IV. Carnrot, C., Vogel, S.R., Byun, Y., Wang, L., Tjarks, W., Eriksson, S., Phipps, A.J. (2006) Evaluation of *Bacillus anthracis* thymidine kinase as a potential target for the development of antibacterial nucleoside analogs. *Biol Chem* (In press).

V. Carnrot, C., Wang, L., Eriksson, S. Molecular characterization of thymidylate kinase from *Bacillus anthracis*. *Manuscript*.

Abbreviations

Enzymes

dAK	deoxyadenosine kinase
dCK	deoxycytidine kinase
dGK	deoxyguanosine kinase
dNK	deoxyribonucleoside kinase
HSV-1 TK	Herpes Simplex virus type 1 thymidine kinase
NDPK	nucleoside diphosphate kinase
NMPK	nucleoside monophosphate kinase
RNR	ribonucleotide reductase
TK	thymidine kinase
TMPK	thymidylate kinase, thymidine monophosphate kinase
UMPK	uridylate kinase, uridine monophosphate kinase

Bases, nucleosides and nucleotides

A, Ado, dAdo	adenine, adenosine, 2'-deoxyadenosine
C, Cyd, dCyd	cytosine, cytidine, 2'-deoxycytidine
G, Guo, dGuo	guanine, guanosine, 2'- deoxyguanosine
T, dThd	thymine, 2'-deoxythymidine
U, Urd, dUrd	uracil, uridine, 2'-deoxyuridine
-MP, -DP, -TP	5'-mono-, 5'-di, 5'-triphosphate of nucleosides

Nucleoside analogs

AraC	1-D-arabinofuranosylcytosine, Cytarabine
AraT	1-D-arabinofuranosylthymine
AZT	3'-azido-2',3'-dideoxythymidine, Zidovudine
d4T	2',3'-didehydro-2',3'-dideoxythymidine, Stavudine
dFdC	2',2'-difluoro-2'-deoxycytidine, Gemcitabine, Gemzar
FCPU	3'-fluoro-5-cyclopropyl-2'-deoxyuridine
5-FdCyd	5-fluoro-2'-deoxycytidine
5-FdUrd	5-fluoro-2'-deoxyuridine, Floxuridine, FUDR
FIAU	1-(2-deoxy-2-fluoro-D-arabinofuranosyl)-5-iodouracil
FLT	3'-fluoro-2',3'-dideoxythymidine
FMAU	1-(2-deoxy-2-fluoro-D-arabinofuranosyl)-5-methyluracil
5-FU	5-fluorouracil, Flurablastin
-MP, -DP, -TP	5'-mono-, 5'-di, 5'-triphosphate of nucleoside analogs

Introduction

Synthesis of DNA precursors

DNA is the central inheritance of all life forms (except of some viruses which have RNA as gene pool instead). To synthesize and maintain DNA the building blocks, i.e. deoxynucleoside triphosphates (dNTPs) are needed. These DNA precursors are synthesized by either the *de novo* or the salvage pathways. In the *de novo* synthesis ribose-5'-phosphate and other small molecules such as amino acids and CO_2 are used to synthesize ribonucleoside monophosphates (NMPs) in multiple reactions catalyzed by different enzymes. These NMPs will be further phosphorylated to ribonucleoside diphosphates (NDPs), which are then reduced by ribonucleotide reductase (RNR) to deoxyribonucleoside diphosphates (dNDPs). A third phosphorylation produces deoxyribonucleoside triphosphates (dNTPs) that are used in DNA replication. The *de novo* synthesis is primarily regulated by ribonucleotide reductase (RNR), an enzyme both allosterically and cell cycle regulated (Elledge, Zhou & Allen, 1992; Greenberg & Hilfinger, 1996; Reichard, 1988). The end products (dNTPs) are responsible for the feedback inhibition of the activity of RNR (Eriksson *et al.*, 1997), while the short half-life of one of the subunits of RNR (called R2) makes the enzyme S-phase regulated (Chabes & Thelander, 2000; Eriksson *et al.*, 1984). However, recently an additional p53 inducible R2 subunit of RNR (p53R2) was identified in mammals (Nakano *et al.*, 2000; Tanaka *et al.*, 2000). Håkansson, Hofer & Thelander (2006) have reported that p53R2 is constitutively expressed in mammalian cells and that p53R2, together with low levels of the other subunit in RNR (R1), may be able to supply a basal level of dNTPs needed for DNA repair and mitochondrial DNA synthesis in resting or non-proliferating cells.

The other pathway, the salvage synthesis occurs in all cells and complements the *de novo* synthesis. Here, deoxyribonucleosides are used as start material, originating from degraded DNA inside or outside the cell and there are specific nucleoside transporters that facilitate the uptake of free deoxyribonucleosides (Cass *et al.*, 1999; Kong, Engel & Wang, 2004; Plagemann, Wohlhueter & Woffendin, 1988). Otherwise, the half-life of free deoxyribonucleosides outside a cell is very short which means that the salvage pathway acts as a recycle station. The first phosphorylation of deoxyribonucleosides to dNMPs is catalyzed by deoxyribonucleoside kinases (dNKs), which traps the deoxyribonucleosides inside the cells. This step is often considered as the rate-limiting step in the salvage pathway. Further phosphorylations towards dNTPs are carried out by different nucleoside monophosphate kinases (NMPKs) and a ubiquitous nucleoside diphosphate kinase (NDPK) (Janin *et al.*, 2000; Van Rompay, Johansson & Karlsson, 2000). Recycling of free purine and pyrimidine bases is also part of the salvage pathway, but is not the focus of this thesis and will not be discussed further. A simplified overview of the nucleotide metabolism is shown in figure 1.

The *de novo* and salvage synthesis of nucleotides exist in almost all organisms from humans to bacteria. *Bacillus anthracis*, one of the pathogenic bacteria this

thesis focuses on, possesses both the *de novo* and salvage pathways. However, the other bacterium in focus, the human pathogen *Ureaplasma urealyticum*, does not have the *de novo* synthesis of purine and pyrimidine bases and no gene for RNR has been identified in its genome (Glass *et al.*, 2000). Therefore, it has to rely completely on the salvage pathway for production of DNA precursors.

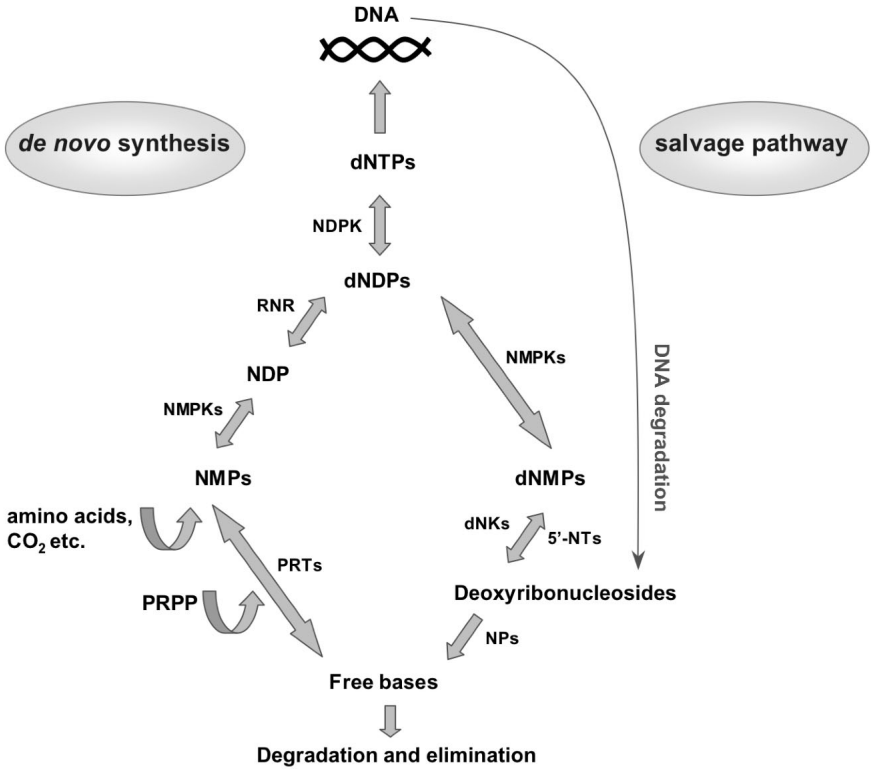


Figure 1. Simplified overview of the synthesis of DNA precursors. dNKs, deoxynucleoside kinases; NDPK, nucleoside diphosphate kinase; NMPKs, nucleoside monophosphate kinases; NPs, nucleoside phosphorylases; 5'-NTs, 5'-nucleotidases; PRPP, phosphoribosyl-pyrophosphate; PRTs, phosphoribosyl-transferases; RNR, ribonucleotide reductase.

Bacteria in focus

Bacillus anthracis

Bacillus anthracis (*B. anthracis*) is a spore-forming gram-positive rod-shaped bacterium that causes anthrax. It belongs to the *Bacillus cereus* group and it is the only obligate pathogen within the *Bacilli*. The virulence of *B. anthracis* is mainly due to its two plasmids, pXO1 and pXO2. pXO1 encodes a tripartite toxin consisting of lethal factor (LF), edema factor (EF) and protective antigen that in itself is non-toxic but is responsible for transporting LF and EF into the host cell.

pXO2 carries the genes for a capsule that contains the anti-phagocytic poly-D-glutamic acid, which makes the cells resistant to ingestion by macrophages (Baillie, 2005). Both plasmids are required for full virulence. An example of such a strain is the Ames strain. In this thesis experimental work has been carried out with an attenuated strain for safety reasons, the Sterne strain, which only has the pXO1 plasmid (Spencer, 2003).

There are three different kinds of anthrax; cutaneous, inhalation and the very rare gastrointestinal form. Spores entering through the skin or mucosa cause the infection. Macrophages ingest the spores but are unable to degrade them. Instead germination occurs into vegetative cells, followed by extracellular multiplication and production of capsules and toxins. The cells escape to the lymph and blood and fatal bacteremia follows (Mock & Fouet, 2001; Öncü, Öncü & Sakarya, 2003). Cutaneous anthrax is the most common form and all three forms are usually caused by the handling of infected animals or their products. The prognosis of cutaneous anthrax is good and treatment is not always necessary compared to inhalation and gastrointestinal anthrax, which have a mortality rate >80% (Baillie & Read, 2001).

Because the bacterium forms spores that are heat-resistant *B. anthracis* is regarded as an attractive bioweapon. *B. anthracis* has been used by several nations and groups throughout the years and most recently in the Gulf War (Zilinskas, 1997) and the postal attack incidents in USA after 9/11 2001. Though, the largest outbreak caused by inhalation anthrax, occurred by mistake in Sverdlovsk (former Soviet Union) after an explosion in a biological laboratory. Between 64 and 105 people died of the disease (Spencer, 2003; Öncü, Öncü & Sakarya, 2003).

The regular treatment of inhalation anthrax is antibiotics (e.g. ciprofloxacin), as is prophylaxis, with e.g. fluoroquinolones and doxycycline. There are also vaccines available as well as ongoing research on new vaccines, but they all suffer from problems such as associated transient side effects and relatively high expense of production (Baillie, 2005; Spencer, 2003).

Ureaplasma urealyticum

Ureaplasma urealyticum (*parvum* biovar) (*Uu*) is a human pathogen that colonizes the mucosa of the urogenital tract. *Uu* belongs to the class of Mollicutes, which are considered as the smallest known free-living organisms. Mollicutes do not have a cell wall but belong phylogenetically to the gram-positive bacteria (Glass*, et al.*, 2000; Krause & Taylor-Robinson, 1992). *Ureaplasma spp*. differ from other mollicutes in having a urease that accounts for 95% of its energy supply. The urease hydrolyzes urea to ammonia and CO_2, which is associated with ATP generation (Glass*, et al.*, 2000; Pollack, 2001).

Uu is an opportunistic pathogen found in 65-80% of adults and is the most common cause of non-chlamydial non-gonococcal urethritis (Pollack, 2001). Additionally, *Uu* is associated with complications in relation to pregnancy such as preterm births, infertility, spontaneous abortions, chorioamnionitis and infections

in newborns (Casell *et al.*, 1993). Pneumonia, chronic lung disease, also known as bronchopulmonary dysplasia, and meningitis are examples of diseases that can arise from *Uu* present in the lungs, the central nervous system or systemically in neonates (Hudson & Talbot, 1997; Kotecha *et al.*, 2004; Schelonka *et al.*, 2005). Numerous mechanisms have been proposed for infections induced by *Uu*. For more information about pathogenic mechanisms, see recent reviews (Skevaki & Kafetzis, 2003; Waites, Katz & Schelonka, 2005).

Neonatal *Uu* infection is usually treated with erythromycin (Skevaki & Kafetzis, 2003). However, there are ongoing discussions whether erythromycin is effective. Newer agents belonging to the macrolides are possible candidates in treatment of infected preterm infants, but more studies and treatment trials need to be performed before any conclusions can be drawn (Waites, Katz & Schelonka, 2005).

Enzymes in deoxyribonucleoside salvage

Deoxyribonucleoside kinases (dNKs)

As mentioned earlier dNKs are the enzymes catalysing the initial phosphorylation of deoxyribonucleosides in the salvage pathway and an overview of the pathway is seen in figure 2. There are different sets of dNKs in many organisms. Thymidine kinase (TK) is found almost universally from viruses to humans, while other dNKs vary in their presence in different species. In humans there are four dNKs with overlapping specificities; cytosolic thymidine kinase (TK1) and deoxycytidine kinase (dCK), and mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK). *Escherichia coli* (*E. coli*) on the other hand has only one dNK, a TK (Karlström, 1970; Okazaki & Kornberg, 1964a). Another bacterium with known dNK genes and activities are *Bacillus subtilis*, that has a TK, deoxyadenosine deoxycytidine kinase (dAK/dCK) and a dGK (Andersen & Neuhard, 2001; Møllgaard, 1980). Similarly *Lactobacillus acidophilus* (*L. acidophilus*) has a TK, dAK/dCK and a dAK/dGK. Note that *L. acidophilus* has no *de novo* synthesis and therefore has to rely on its salvage enzymes for its DNA precursor synthesis (Ives & Ikeda, 1998). Yeast, on the otherhand, has no deoxyribonucleoside salvage enzymes (Cherry *et al.*, 1998). Another example of the diversity of dNKs is shown in the fruit fly (*Drosophila melanogaster*) and the mosquito (*Anopheles gambiae*), which both have a single dNK that phosphorylates all four deoxyribonucleosides (Johansson *et al.*, 1999; Knecht *et al.*, 2003; Munch-Petersen, Piskur & Søndergaard, 1998).

In *mycoplasmas* two dNKs are present, a TK and a dAK. This thesis partly focuses on TK from *Uu* and Wang *et al.* (2001) have previously characterized dAK from *Mycoplasma mycoides*, subspecies *mycoides* small colony type. *B. anthracis* shows a slightly different pattern having a TK, a dGK-like and a dAK/dCK-like kinase. The TK is described in this thesis but no detailed studies have been done so far regarding cloning and characterization of the two latter enzymes.

dNKs often show similar substrate specificity between organisms even though the amino acid sequence identities are low. How can they be so dissimilar in amino acid sequence and still have very similar function? The answer lies in the three dimensional (3D) structure. The crystal structures of human dCK and dGK, dNK from fruit fly and TK from Herpes simplex virus all have a similar 3D-fold (Brown *et al.*, 1995; Johansson *et al.*, 2001; Sabini *et al.*, 2003; Wild *et al.*, 1995). However, the recently determined structures of human TK1 and TK from *Uu* (Welin *et al.*, 2004) show a completely different fold from the other dNKs. Given these results it has been proposed to divide the dNKs into two families or groups, a dCK/dGK/dNK-group and a TK1-group (Sandrini & Piskur, 2005).

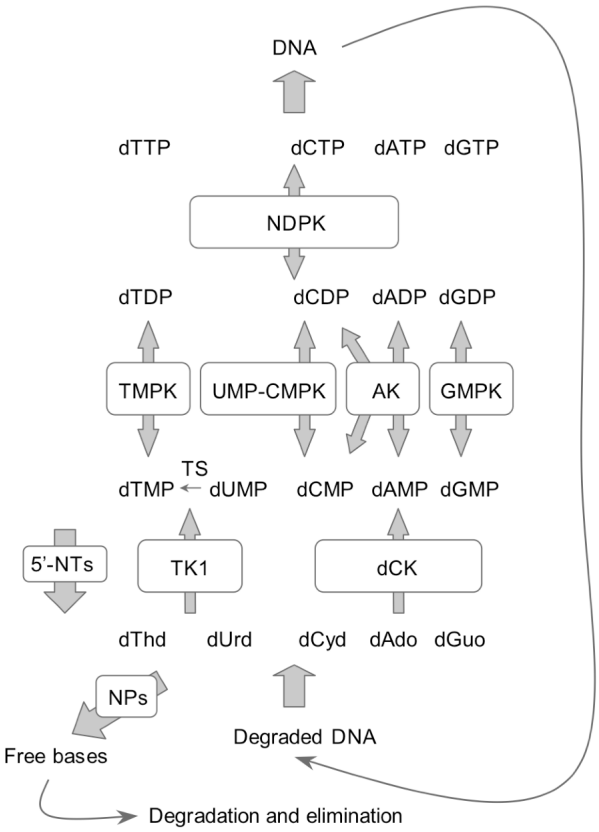


Figure 2. Simplified overview of the salvage pathway of DNA precursors. AK, adenylate kinase; dCK, deoxycytidine kinase; GMPK, guanylate kinase; NDPK, nucleoside diphosphate kinase; NPs, nucleoside phosphorylases; 5'-NTs, 5'-nucleotidases; TK1, cytosolic thymidine kinase; TMPK, thymidylate kinase; TS, thymidylate synthase; UMP-CMPK, uridylate-cytidylate kinase. In mammals there is one UMP-CMPK with dual specificity and in bacteria there are two separate enzymes, a UMPK and a CMPK. The phosphorylation of dCyd, dAdo and dGuo are made by one to three different dNKs in different organisms.

Nucleoside monophosphate kinases (NMPKs)

NMPKs catalyze the reversible phosphorylation of nucleoside monophosphates to nucleoside diphosphates (figure 2). Apart from the dNKs, NMPKs both belong to the salvage and the *de novo* pathways (figure 1). Hence, NMPKs are excellent targets for drug design, since inhibition of NMPKs will knock out both pathways for DNA precursor synthesis. For example, all the NMPKs in *Mycoplasma genitalium* are essential for organism survival as demonstrated in a recent study using global transposon mutagenesis (Glass *et al.*, 2006). These enzymes are usually base specific and less specific for the sugar. Generally, there is at least one enzyme for each base, with a combined UMP-CMP kinase in eukaryotes and two separate enzymes in prokaryotes. In humans a thymidylate kinase (TMPK), a UMP-CMP kinase and several adenylate and guanylate kinases are found (Ren *et al.*, 2005; Van Rompay, Johansson & Karlsson, 2000). *Uu* and *B. anthracis* have open reading frames coding for five different NMPKs (one per base) but there are no publications yet about any NMPK from either bacterium. All NMPKs belong to the nucleoside monophosphate kinase family except the prokaryotic UMPKs that instead belong to the amino acid kinase family (Serina *et al.*, 1995; www.sanger.ac.uk//cgi-bin/Pfam/getacc?PF00696; 11-Sep-2006).

Among the diversity and variety of dNKs and NMPKs there are a few things they have in common that are worth special attention. First, although ATP is considered as the physiological phosphate donor dNKs and NMPKs are usually not limited in their choice of phosphate donors. Other nucleoside triphosphates can in some cases even be better phosphate donors *in vitro* as compared with ATP. Second, Mg^{2+} is required for the reaction to take place with any kinase; and last, the end product, i.e. the triphosphate form of the phosphate acceptor, is usually a feedback inhibitor of the reaction.

Nucleoside diphosphate kinase (NDPK)

NDPK is a ubiquitous enzyme, which phosphorylates nucleoside diphosphates to their corresponding triphosphates. The enzyme is extraordinary in that it is unspecific regarding both phosphate acceptor and donor, i.e. NDPK uses the ribose and deoxyribose forms of both purine and pyrimidine nucleotides as substrate. NDPK has also been suggested to be a multifunctional protein involved in e.g. cell growth and differentiation and tumour metastasis in relation to signal transduction (Bernard *et al.*, 2000; Kimura *et al.*, 2000). There are several 3D structures of NDPK from different species (Besir *et al.*, 2005; Chen *et al.*, 2002; Chiadmi *et al.*, 1993; Dumas *et al.*, 1992; Webb *et al.*, 1995) and the enzyme is highly conserved both in the overall fold and in the active site. In humans there are eight NDPK genes with tissue specific expression and different subcellular localization of the proteins (Lacombe *et al.*, 2000). The two major isoforms are A and B, which are almost 90% identical (Gilles *et al.*, 1991), whereas bacteria have one single gene (e.g. *B. anthracis*). No *ndk* gene has been found in the genome of *Uu* (Glass*, et al.*, 2000) and other glycolytic enzymes have been suggested to play a role in nucleotide synthesis (Pollack *et al.*, 2002).

The work in this thesis concerns only the first two steps in the salvage pathway, more specifically the thymidylate synthesis, whose enzymes now will be described in more detail.

Thymidine kinase (TK)

TK is a well-known and widely distributed dNK. TK catalyzes the irreversible phosphorylation of dTMP to dTDP. Already in 1964 purification and characterization of TK from *E. coli* was published (Okazaki & Kornberg, 1964a; Okazaki & Kornberg, 1964b). Investigations on viral TKs followed (Carp, 1967; Kit, Dubbs & Anken, 1967; Klemperer *et al.*, 1967; Littlefield & Basilico, 1966; Sheinin, 1966) and Herpes Simplex virus type 1 TK (HSV-1 TK) is now one of the most investigated TKs, largely due to its broad substrate specificity and usefulness in commercial drug design. HSV-1 TK belongs to the dCK/dGK/dNK family, which is not the focus of this thesis and will not be discussed further. For more information on HSV-1 TK see reviews (Fillat *et al.*, 2003; Waldman, Haeusslein & Milman, 1983). TKs from poxviruses and Vaccinia virus on the other hand belong to the TK1 family. An alignment including Vaccinia virus is shown in figure 3 that illustrates the conservation of TK between organisms.

Bacterial TKs

TK from *E. coli* (*Ec*-TK) was the first and the only bacterial TK that has been purified and characterized. *Ec*-TK phosphorylated deoxythymidine (dThd), deoxyuridine (dUrd) and some 5'-halogenated dUrd analogs. ATP and dGTP were the preferred phosphate donors and dTTP was a feedback inhibitor. Nucleoside di- and triphosphates were activators with dCDP being the most effective one in stimulating the dThd phosphorylation (Okazaki & Kornberg, 1964a; Okazaki & Kornberg, 1964b). dTTP and the activators affected the subunit interaction of *Ec*-TK so the enzyme changed quarternary structure to a higher molecular weight. This change also made *Ec*-TK less sensitive for temperature changes regarding enzyme activity (Iwatsuki & Okazaki, 1967a; Iwatsuki & Okazaki, 1967b). In addition to these early studies, there are a couple of screening studies of TK activities in microorganisms (Saito & Tomioka, 1984; Saito, Tomioka & Ohkido, 1985), which illustrate a wide distribution of TK in different bacteria. Microbial genome sequencing projects now reveal that many bacteria species possess a *tdk* gene and table 1 summarizes the presence of the *tdk* gene in known bacterial genomes. Most classes of bacteria contain genera that have a *tdk* gene even if there are more genera in total that do not posess the gene. The variability of gene presence between the classes and orders is high and phylogenetic relationships, natural environment, host factors and variations in nucleotide metabolism can probably explain these distributions. Further biochemical studies will most likely provide information regarding why some bacteria have and some lack the *tdk* gene.

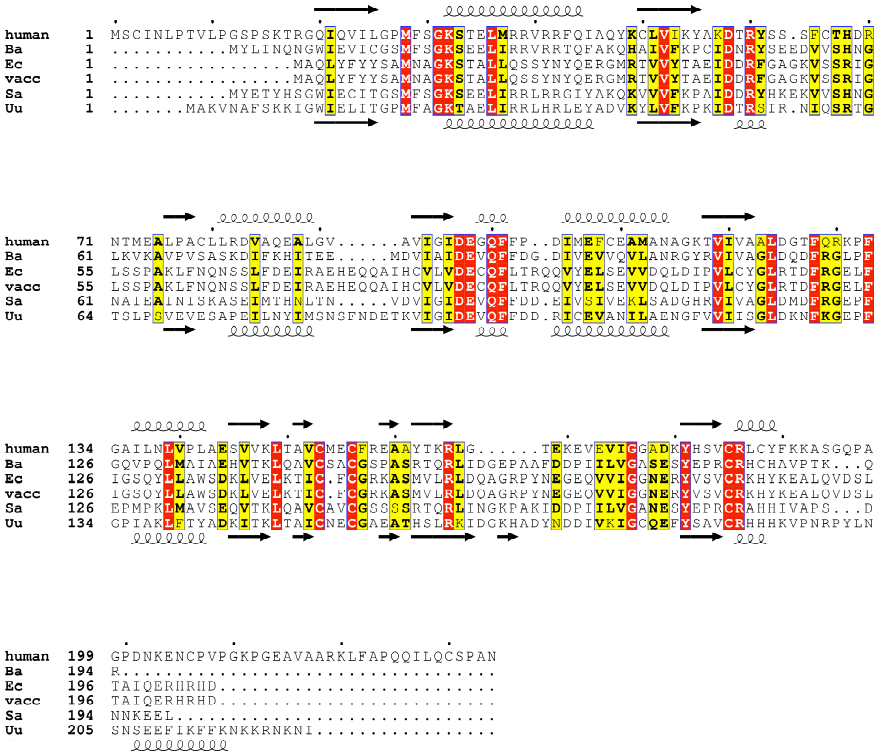


Figure 3. Alignment of TK1 sequences from human (P04183), *B. anthracis* (AAT57468), *E. coli* (NP_287483.1), Vaccinia virus (AAB96503.1), *Staphylococcus aureus* (ABD37699) and *U. urealyticum* (NP_078433) with secondary structure elements from human TK1 (PDB code: 1XBT) and TK from *U. urealyticum* (PDB code: 2B8T) above and under the alignment, respectively. Dots above the alignments mark every tenth residue, human TK1 numbering. Identical residues in all sequences are marked in red and similar residues are marked in yellow. The alignment was done using ClustalW and the figure was generated in WebESPript (v. 2.2).

Table 1. Presence of the *tdk* gene in bacteria with known genomes.

Class of bacterium	*General presence of a tdk gene*	*Ratio of exceptions at genus level*
Actinobacteria	No	4/20 (31)
Bacteroidetes/Chlorobi	Yes/No	3/6 (10)
Bacillales	Yes	3/8 (59)
Chlamydiae	No	0/3 (11)
Clostridia	No	3/8 (10)
Cyanobacteria	No	0/8 (19)
Lactobacillales	Yes	3/7 (28)
Mollicutes	Yes	0/3 (16)
Spirochaetes	No	1/3 (10)
Others	No	2/13 (16)
Proteobacteria (phylum) α to ε class γ class	No	9/72 (124)
Order of bacterium in the γ class		
Enterobacteriales	Yes	3/9 (33)
Pasteurellales	Yes	1/4 (7)
Pseudomonadales	No	0/2 (12)
Vibrionales	Yes	0/2 (13)
Xanthomonadales	Yes/No	1/2 (8)
Others	No	7/26 (32)

Source: TIGR CMR database (http://cmr.tigr.org/tigr-scripts/CMR/shared/Annotation Search.cgi; 04-Oct-2006) and NCBI databases (www.ncbi.nlm.nih.gov/sutils/genom_ table.cgi; 04-Oct-2006). The *tdk* gene was searched for as keyword ("thymidine kinase") in TIGR CMR and the list of all fully sequenced bacterial genomes was obtained from NCBI. The genus exceptions are defined as having a *tdk* gene when the gene is not generally present in the class (or order) the genus belongs to, and vice versa. The ratio is determined according to how many genera there are in each class (or order). The digits in brackets are the total number of fully sequenced genomes in the class or order. Taxonomy classification according to the taxonomy browser of NCBI.

Human TK1

Human TK1 (hTK1) (EC 2.7.1.21) is a cytosolic enzyme and has homologues in most organisms. Its gene is located on chromosome 17 (McDougall, Kucherlapati & Ruddle, 1973; Petty *et al.*, 1996) and the hTK1 cDNA codes for a polypeptide of 234 amino acids with a molecular weight of 25.5 kDa (Bradshaw & Deininger, 1984; Munch-Petersen *et al.*, 1991; Sherley & Kelly, 1988). The expression of hTK1 is cell cycle regulated and the enzyme level is at its peak in S-phase, which then falls to barely detectable levels in the G1 phase (Bello, 1974; Coppock & Pardee, 1987; Kauffman & Kelly, 1991). hTK1 is degraded via a ubiquitin-proteasome pathway after mitotic exit and the ubiquitin ligase complex APC/C-Cdh1 targets hTK1 by recognizing a KEN box in the C terminal region (Ke & Chang, 2004; Ke *et al.*, 2005). Detection of the hTK1 level in serum is useful in cancer diagnostics where cancer patients have elevated levels of hTK1 (Gronowitz *et al.*, 1984; Hallek *et al.*, 1992; Li *et al.*, 2005; Zhang *et al.*, 2006). In addition, specific hTK1 antibodies have recently been developed, which may be beneficial in future diagnostics and clinical use (He *et al.*, 2000; Kuroiwa *et al.*, 2001; Wu *et al.*, 2003; Zhang *et al.*, 2001).

hTK1 is strictly pyrimidine specific and phosphorylates only dThd and dUrd. In addition several dThd and dUrd analogs can be phosphorylated. The chemical structure of dThd and the numbering of the atoms ihTs shown in figure 4. Analogs with small modifications at the 5-position (e.g. 5-fluoro, 5-ethyl) are good substrates whereas with bulkier or more polar substitutions the activity decreases drastically (e.g. 5-propenyl, 5-bromovinyl) (Eriksson *et al.*, 1991; Johansson & Eriksson, 1996; Lee & Cheng, 1976; Munch-Petersen*, et al.*, 1991). Modifications at the N3-position on the base decrease the activity but still these analogs are reasonably good substrates for hTK1 (Al-Madhoun, Tjarks & Eriksson, 2004; Bandyopadhyaya *et al.*, 2005). Analogs with 3'-substitutions on the sugar have different acceptability. For example, AZT (3'-azidodeoxythymidine) and FLT (3'-fluorodeoxythymidine) show substantial activity while d4T (2',3'-didehydrodeoxythymidine) is a very poor substrate for hTK1 (Balzarini, Herdewijn & De Clercq, 1989; Furman *et al.*, 1986; Johansson & Eriksson, 1996; Matthes *et al.*, 1988). Ribosyl and arabinosyl sugars are also very poorly recognized by hTK1 with the exceptions of FIAU (2'-fluoro-arabinosyl-5-iodo-deoxyuridine) and FMAU (2'-fluoro-arabinosyl-5-methyl-deoxyuridine) (Al-Madhoun, Tjarks & Eriksson, 2004).

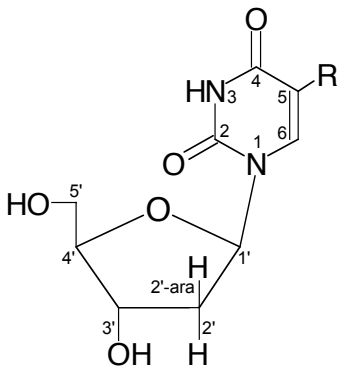


Figure 4. The chemical structure of dThd or dUrd. The numbers 1' to 5' is used to name the location of modifications on the deoxyribose carbon atoms in nucleoside analogs. 2'-ara, 2'-arabinosyl. The numbers 1 to 6 show the corresponding numbering for the atoms in the base moiety. dThd, R= CH_3; dUrd, R= no atom group.

Regarding phosphate donors hTK1 can use all nucleoside triphosphates as donors, except dTTP, but ATP and dATP are by far the most preferred ones (Eriksson *et al.*, 2002). dTTP is a feedback inhibitor implicated in the regulation of the dTTP level and thereby the dNTP pool in the cell. ATP can also regulate hTK1 activity *in vitro*. In the presence of ATP hTK1 transforms from a low affinity dimer to a high affinity tetramer. This is a slow process and it is dependent on the enzyme concentration. The resulting tetrameric form of hTK1 has a 20-fold lower apparent K_m value compared to the dimeric form, in the absence of ATP (Li *et al.*, 2004; Munch-Petersen, Tyrsted & Cloos, 1993; Munch-Petersen *et al.*, 1995).

Thymidine monophosphate kinase (TMPK)

TMPK catalyzes the reversible phosphorylation of dTMP to dTDP. This reaction is at the junction where the salvage and *de novo* synthesis meet. Therefore, TMPK can be a potential drug target since this enzyme affects both pathways in dTTP synthesis.

Several TMPKs from different organisms have been investigated throughout the years, such as human, yeast, *Mycobacterium tuberculosis* (*M. tuberculosis*), *Streptococcus pneumoniae*, *E. coli*, *Yersinia pestis* and Vaccinia virus. Figure 5 shows an alignment of TMPKs from different organisms, which demonstrates a relatively low degree of amino acid conservation. Nonetheless all known TMPKs are dimers and share the characteristic of being pyrimidine specific and sensitive for substrate modifications. They also share the ability to use other phosphate donors than ATP (Brundiers *et al.*, 1999; Chenal-Francisque *et al.*, 1999; Jong & Campbell, 1984; Lavie *et al.*, 1998a; Lavie *et al.*, 1998b; Lee & Cheng, 1977; Munier-Lehmann *et al.*, 2001; Petit & Koretke, 2002; Topalis *et al.*, 2005).

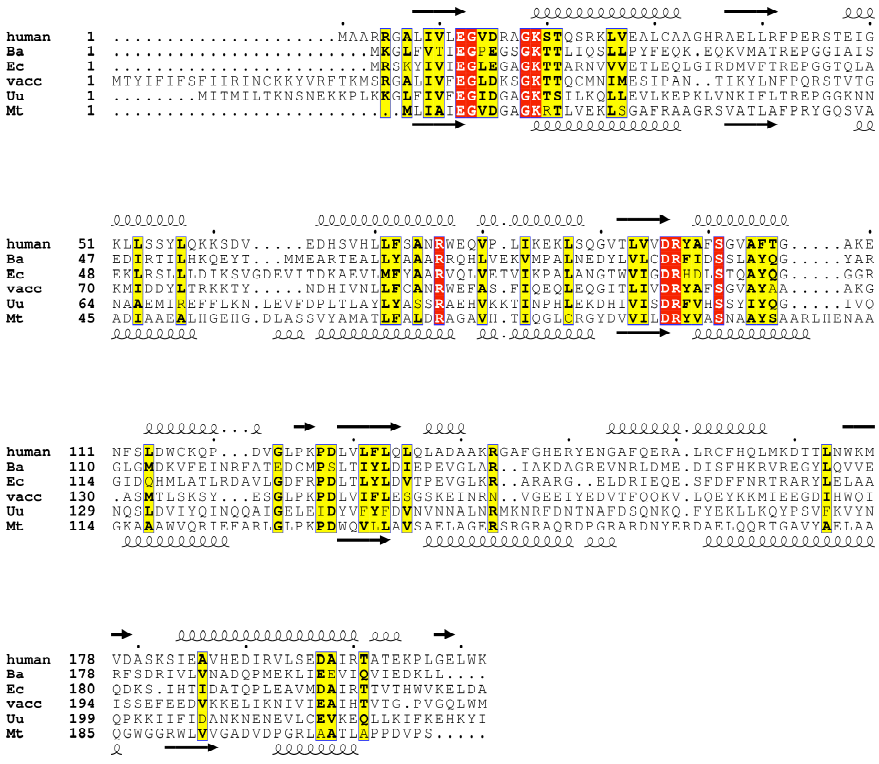


Figure 5. Alignment of TMPK sequences from human (NP_036277), *B. anthracis* (YP_026316), *E. coli* (AP_001724), Vaccinia virus (AAR18016), *U. urealyticum* (AAF30425) and *M. tuberculosis* (NP_217764) with secondary structure elements from human TMPK (PDB code: 1XBT) and TMPK from *M. tuberculosis* (PDB code: 13GU)

above and under the alignment, respectively. Dots above the alignments mark every tenth residue, human TMPK numbering. Identical residues in all sequences are marked in red and similar residues are marked in yellow. The alignment was done using ClustalW and the figure was generated in WebESPript (v. 2.2).

Bacterial TMPKS

TMPK from *M. tuberculosis* (*Mt*-TMPK) is one of the most studied TMPKs, due to its catalytic mechanism that is proposed to be unique among the known TMPKs and its potential as a drug target because of the upcoming multiresistance against the bacterium. A magnesium ion is supposed to compensate for a missing positively charged residue in the active site seen in other TMPKs, and thereby coordinating the phosphate acceptor (Fioravanti *et al.*, 2003; Haouz *et al.*, 2003; Li de la Sierra *et al.*, 2001). This difference is also supposed to explain why AZTMP is a substrate for other TMPKs, even if sometimes poorly, and a competitive inhibitor with *Mt*-TMPK (Fioravanti *et al.*, 2005; Munier-Lehmann, *et al.*, 2001). Other studies have shown that dTMP analogs substituted at the 3' and 2' position of the ribose and at the 5-position on the base are the best leads for antibacterial drug development (Haouz, *et al.*, 2003; Vanheusden *et al.*, 2003a; Vanheusden *et al.*, 2002; Vanheusden *et al.*, 2003b). Studies with bicyclic nucleosides as inhibitors that are to be published this year are also promising compounds to use against *Mt*-TMPK (Van Daele *et al.*, 2006).

TMPK of *E. coli* (*Ec*), *Yersinia pestis* (*Yp*) and *Streptococcus pneumoniae* (*Sp*) have also been studied. *Ec*-TMPK was purified and characterized already in the 1960's, where the enzyme was found to phosphorylate dTMP, dUMP and 5-IdUMP with ATP and dATP as the preferred phosphate donors (Nelson & Carter, 1969). *Ec*-TMPK has also been used as a comparing TMPK to the TMPK in question in several studies and has therefore been kinetically characterized a number of times (Chenal-Francisque, *et al.*, 1999; Lavie, *et al.*, 1998a; Munier-Lehmann, *et al.*, 2001; Van Daele, *et al.*, 2006). *Ec*-TMPK and *Yp*-TMPK have high sequence identity (70%) and the two enzymes behave similarly regarding phosphate donor and acceptor specificity, except with AZTMP. Both enzymes have comparable K_m values for AZTMP but differ significantly in V_{max} values. The V_{max} for *Yp*-TMPK is 1% of that of dTMP, while the corresponding value for *Ec*-TMPK is 16% (Chenal-Francisque, *et al.*, 1999). *Sp*-TMPK shares the low substrate specificity for AZTMP with *Yp*-TMPK. In fact, no activity with AZTMP was detected in the study of *Sp*-TMPK (Petit & Koretke, 2002). Moreover, the characterization of *Sp*-TMPK showed that the reaction mechanism of the enzyme was an ordered pathway, in which ATP binding was prior to dTMP binding (Petit & Koretke, 2002).

Human TMPK

Human TMPK (hTMPK) (EC 2.7.4.9) is a cytosolic enzyme and was first described in leukemic leukocytes (Nakai *et al.*, 1966). Its gene is located on chromosome 2 (GenBank®) and hTMPK consists of 212 amino acids with a molecular weight of 23.8 kDa (Lee & Cheng, 1977; Tamiya *et al.*, 1989). The expression of hTMPK is cell cycle regulated and the enzyme is degraded in M and

early G1 phase (Huang *et al.*, 1994; Liang *et al.*, 1995). Similar to hTK1, hTMPK is degraded via the same ubiquitin-proteasome pathway. hTMPK is targeted by either the APC/C-Cdc20 or the APC/C-Cdh1 complex, which recognize a D box or a KEN box, respectively, in the hTMPK sequence (Ke*, et al.*, 2005). Ke and coworkers have also shown that if TMPK and TK are not degraded the dTTP pool increases dramatically and a dNTP pool imbalance follows. In turn, this leads to growth retardation and a strikingly high frequency of gene mutations. Thus, the degradation control of TMPK and TK is essential to maintain a balanced dNTP pool and to prevent genetic instability (Ke*, et al.*, 2005).

hTMPK phosphorylates only dTMP and deoxyuridine monophosphate (dUMP). but several dTMP and dUMP analogs can also be phosphorylated (Lavie & Konrad, 2004). The majority of the analogs that have been tested are modified at the 3' position on the ribose and they have variable activities. d4TMP is a rather good substrate while FLTMP, ddTMP and AZTMP are poor ones (Ostermann *et al.*, 2003). A combination of size and hydrogen bonding abilities of the 3'-substituent, together with its effect on the sugar ring conformation determine the phosphorylation efficiency of the 3'-analog by hTMPK (Lavie & Konrad, 2004). FMAUMP has also proven to be a substrate analog for hTMPK. The D-isomer of FMAUMP is more efficiently phosphorylated than the L-isomer and their efficiencies are about 75% and 1%, respectively, in comparison with dTMP, which clearly reflects the discrimination of L-nucleosides by hTMPK (Hu *et al.*, 2005).

Regarding phosphate donors, hTMPK prefers ATP and dATP but can also use GTP and dGTP. Other (deoxy)ribonucleotide triphosphates are poor phosphate donors. dTTP inhibits competitively towards either ATP or dTMP and is considered to be a feed-back inhibitor, being the end product in dTTP synthesis (Lee & Cheng, 1977).

Structures of thymidine kinase (TK) and thymidine monophosphate kinase (TMPK)

TK

The first structures of a TK belonging to the TK1 family were determined and published in collaboration with H. Eklund's group (SLU, Uppsala, Sweden) in 2004 and are presented in this thesis. Today, there are five solved structures in different complexes from three different organisms; human, *Uu* and *Clostridium acetobutylicum* (Birringer *et al.*, 2005; Kosinska *et al.*, 2005; Welin*, et al.*, 2004; www.nesg.org; 13-Sep-2006). The TKs are tetramers and each monomer consists of a six-stranded parallel β-sheet surrounded by four helices and a long flexible loop (figure 6). Moreover, the monomer comprises one core domain (the α/β domain) and a much smaller lasso- and zinc-containing domain. The core domain includes the indispensable P-loop and is similar to the ATP-binding domain in enzymes of the RecA-F_1ATPase family. The lasso- and zinc-domain on the other hand does not resemble any known structural domain. The lasso covers the substrate binding site and is kept in place by a conserved arginine-tyrosine couple in two perpendicular β ribbons. In turn, four cysteines in the β-ribbons coordinate

the zinc ion. The last of the cysteines is replaced by a histidine in *Uu*. Between these two domains the active site is formed in a cleft. The phosphate binding site with the P-loop is more exposed to the environment.

(A) **(B)**

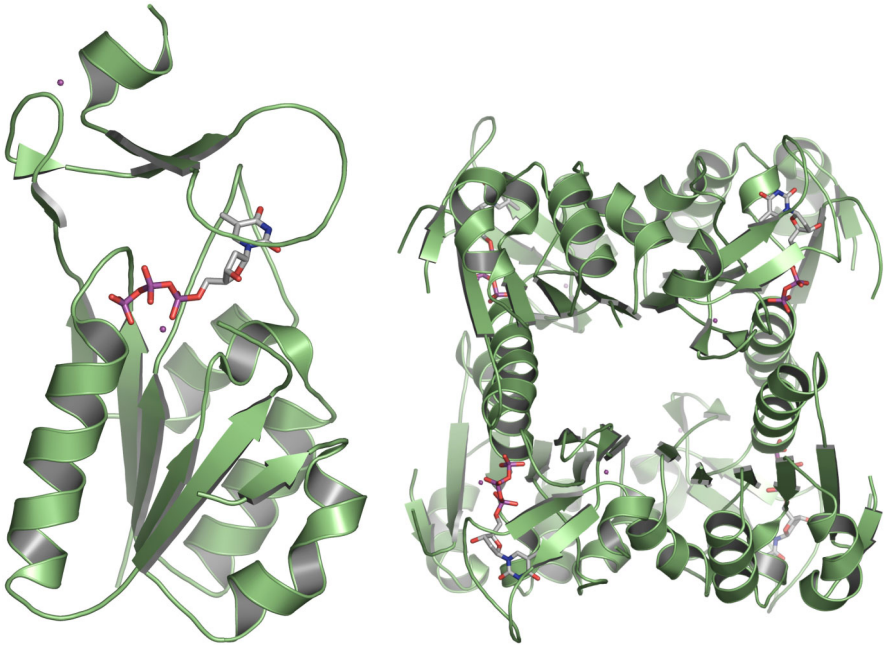


Figure 6. Structures of human TK1 (hTK1), PDB code: 1XBT. (A) Monomer and (B) dimer of hTK1, with dTTP, Mg^{2+} and Zn^{2+}.

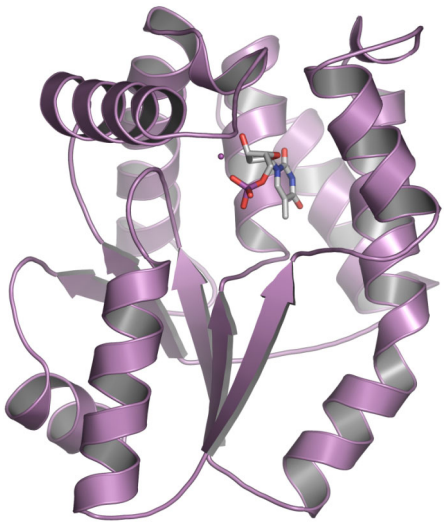
The two hTK1 structures were solved almost simultaneously in two different labs. Both enzymes were truncated in their C-termini and one of them also lacked 15 amino acids in its N-terminal. dTTP is found in the active site in both structures binding backwards with the triphosphates to the P-loop. Almost all interactions between dTTP and the lassos in the enzymes are main-chain interactions (Birringer, *et al.*, 2005). Birringer, *et al.* (2005) assumed lysine 32 to be responsible for the phosphate transfer from ATP and glutamate 98 to aid in the catalysis by binding to the substrate. This role of the glutamate was later on confirmed in the *Uu*-TK structure in complex with dThd where the glutamate is hydrogen bonded and closer to the 5'-oxygen compared to when dTTP is bound (Kosinska, *et al.*, 2005). The third structure complex, TK from *Clostridium acetobutylicum*, has no substrate or nucleoside moiety bound but ADP instead that binds in the donor site. With an empty active site the lasso becomes disordered as is seen in this structure (www.nesg.org; 13-Sep-2006).

TMPK

Crystal structures of TMPK in different complexes have been solved from five organisms to date; human, yeast, *E. coli*, *M. tuberculosis* and *Staphylococcus aureus* (*S. aureus*) (Kotaka *et al.*, 2006; Lavie, *et al.*, 1998a; Lavie, *et al.*, 1998b; Li de la Sierra, *et al.*, 2001; Ostermann *et al.*, 2000b). TMPKs are dimers and each monomer is composed of eight to nine α-helices surrounding a β-sheet core consisting of five β strands (figure 7). The monomer can be divided into three parts; the core region, the substrate binding region and the LID region. The core region is the most conserved part and contains the essential P-loop that, together with the LID region, are involved in the phosphate donor binding. The LID region is a flexible stretch of residues, which upon binding of the phosphate donor closes down over the phosphate binding site.

All TMPKs share the same overall fold but show differences in the active site. Lavie, *et al.* (1998b) have proposed a classification based on interactions at the phosphate binding site. Class I TMPKs, to which human and yeast belong, have a basic residue in the P-loop that interacts with the γ-phosphate of the phosphate donor. Class II TMPKs, where *E. coli* and *S. aureus* are members, have a glycine instead and additional basic residues (mostly arginines) in the LID region that interact with the phosphate donor. TMPK from *M. tuberculosis* (*Mt*-TMPK) is said to have a third binding mechanism that involves a magnesium ion, compensating for a missing positively charged residue in the substrate binding. In addition, the last residue in the P-loop (an arginine) in *Mt*-TMPK plays the same role in phosphate donor binding as the magnesium ion does in human TMPK (Fioravanti, *et al.*, 2003). This classification has its origin in the phosphorylation of AZTMP. Human and yeast TMPKs phosphorylate AZTMP poorly while TMPK from *E. coli* only has a 2.5-fold lower phosphorylation rate with AZTMP compared to dTMP (Fioravanti, *et al.*, 2003; Lavie, *et al.*, 1998b; Lavie *et al.*, 1997; Ostermann *et al.*, 2000a). In contrast, AZTMP is a competitive inhibitor to *Mt*-TMPK (Munier-Lehmann, *et al.*, 2001). The difference in the AZTMP phosphorylation efficiency between human, yeast and *E. coli* TMPKs has been correlated to the interaction of the azido group with a conserved aspartate or glutamate in the P-loop. The aspartate in yeast and human TMPK interacts with the 3'-hydroxyl of dTMP in a bidentate manner. To accommodate the larger azido group in AZTMP a shift of the P-loop occurs. In *E. coli* TMPK the corresponding residue is glutamate that instead interacts in a side-on fashion with the 3'-hydroxyl group of dTMP. This difference results in an insignificant movement of the P-loop in the presence of the azido group (Lavie, *et al.*, 1998b). Thus, a shift of the P-loop is clearly fatal for the phosphorylation capacity in the enzymes. The inhibition mechanism of *Mt*-TMPK is not suggested to involve P-loop movements. Instead, the azido group prevents the binding of the magnesium ion (Fioravanti, *et al.*, 2005).

(A)



(B)

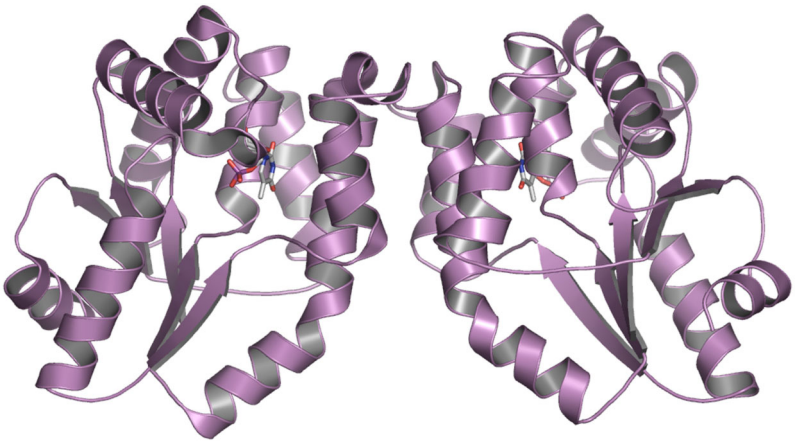


Figure 7. Structures of TMPK from *M. tuberculosis* (*Mt*-TMPK), PDB code: 13GU. (A) Monomer and (B) dimer of *Mt*-TMPK, with dTMP and Mg^{2+}.

Targeting enzymes in the salvage pathway

All organisms need to replicate their genomes in order to survive and the above described pathways are the tools to fulfil that request. There are a number of studies that show the importance of functioning deoxyribonucleoside salvage. Mice lacking TK1 develop kidney failure (Dobrovolsky *et al.*, 2003) and humans with deficiencies in mitochondrial dNKs suffer from liver or multi-systemic failure

(mutated dGK) or severe myopathy (mutated TK2) (Mandel *et al.*, 2001; Saada *et al.*, 2001; Wang *et al.*, 2005). Enzymes in the salvage pathway are also important from a pharmacological point of view. Drugs targeting nucleotide metabolism have been successfully developed against cancer and viral diseases. Most of these compounds are nucleoside analogs, which exhibit their efficacy after they have been activated by cellular enzymes (e.g. dNKs) from their proforms to their triphosphate forms that interfere with viral or cellular DNA synthesis (Darby, 1994; Herdewijn, 1994). The activated nucleoside analogs usually exert their character by introducing DNA strand breakage or chain termination. dFdC (Gemcitabine, Gemzar®) and AraC (Cytarabine) are examples of powerful and commercialized nucleoside analogs used in cancer treatment, which cause chain breakage (Grant, 1998; Grindey, Hertel & Plunkett, 1990). AZT (Zidovudine) (Sperling, 1998), the first approved nucleoside analog against HIV, and the successful antiherpetic analog Acyclovir (Sheffield *et al.*, 2003) are examples of chain terminators. Nucleoside analogs such as 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine (5-FdUrd), targeting thymidylate synthase, are also important anticancer drugs (Goldberg & Gill, 2004; Poorter, Bakker & Veenhof, 1998). Moreover, the level of TK1 in sera of cancer patients has been used as a diagnostic marker, since the higher TK1 level indicates a high degree of rapidly growing cells or malignancy (Gronowitz*, et al.*, 1984; Hallek*, et al.*, 1992; Li*, et al.*, 2005; Zhang*, et al.*, 2006).

This thesis deals with two fundamental enzymes in dTTP biosynthesis: TK and TMPK of two pathogenic bacteria. These two enzymes are involved in both the *de novo* and salvage synthesis of dTTP and are essential for organisms to survive (Glass*, et al.*, 2006; Hutchison *et al.*, 1999; Kobayashi *et al.*, 2003; Mushegian & Koonin, 1996), which means that a knockout of the enzymes would lead to cell death. The question is: can we or should we apply the knowledge of enzymes in DNA precursor synthesis to a new area - develop new agents against Bacteria?

After Fleming discovered penicillin in 1929, humans had an honest chance to fight bacteria for the first time in history. Some years later it looked like humans had won the battle against bacterial infections, but the bacteria stroke back. Antibiotic resistance has been found ever since antibiotics were introduced to medical treatment but the resistance did not become a problem until the late 1970's or early 1980's. Today multi-resistant bacteria are a well-known fact and hospitals are struggling with outbreaks of different multi-resistant strains. Therefore, antibiotics with novel targets are definitely needed to combat these bacteria. The work in this thesis has tried to define and started to explore such targets and the results will hopefully be of interest for future drug design of new antibiotics, as well as an exhaustive knowledge basis of the three kinases in question has been established.

Present investigations

Aim

This thesis intends to increase our knowledge about thymidylate synthesis in two pathogenic bacteria; *Ureaplasma urealyticum* and *Bacillus anthracis*. More specifically, two thymidine kinases and one thymidine monophosphate kinase were molecularly characterized with focus on their substrate specificity. In parallel, purification of the enzymes was carried out for crystallization trials with the aim of solving the 3D structures. The work in this thesis also intends to contribute to future drug design of antibacterial agents.

Results and discussion

Methods used in the present investigations are described in detail in papers I-V in the appendix, and will not be described further in this section.

Paper I: Molecular characterization of thymidine kinase from Ureaplasma urealyticum: nucleoside analogues as potent inhibitors of mycoplasma growth

Ureaplasma urealyticum (*Uu*) is a human pathogen causing urethritis and is associated with complications associated with pregnancy. Mollicutes are unable to synthesize purines and pyrimidines *de novo* and therefore has to rely on their salvage pathway. With this background knowledge we decided to study the pyrimidine salvage of *Uu* to investigate possible targets and agents for antibacterial therapy against *Uu*.

The thymidine kinase in *Uu* (*Uu*-TK) was cloned and expressed in an *E. coli* system. The protein was affinity purified in high amounts (ca 40 mg protein/L). Active *Uu*-TK was a dimer as determined by gel filtration. In contrast to human TK1, ATP had no effect on subunit interactions of *Uu*-TK. *Uu*-TK, phosphorylated only dThd and dUrd, similar to human TK1. dThd showed substrate inhibition at concentrations > 20 μM while dUrd did not saturate the enzyme until the millimolar range. Among the pyrimidine nucleoside analogs 5-FdUrd was the most efficient substrate. The order of relative activity of the other analogs was AZT > d4T > AraT. According to substrate specificity and sequence alignments *Uu*-TK was related to human TK1. Regarding phosphate donors, *Uu*-TK could use all nucleoside triphosphates as donors except dTTP. Kinetic parameters of ATP and GTP revealed ATP to be the most efficient phosphate donor of the two. dTTP showed competitive inhibition towards ATP and non-competitive inhibition towards dThd. Hence, like most end products dTTP feedback inhibited an enzyme earlier in the reaction chain. This broad phosphate donor specificity may play a role together with the narrow phosphate acceptor specificity in balancing the dNTP pool. *Uu* DNA has a high AT content (70-75%) and consequently needs more dATP and dTTP than dCTP and dGTP for DNA

synthesis. The ability of *Uu*-TK to produce dTMP with, in principle any available nucleoside triphosphate would surely conduce to the balance of the dNTP pool.

The salvage of dThd, dCyd, 5-FdUrd and 5-FU was also investigated. The levels of dNDPs and dNTPs were much lower than the levels of dNMPs, which would imply that the production to dNDPs is rate limiting. The incorporation of labelled dNTPs into DNA however was quite high, which may suggest that *Uu* DNA polymerase has a high affinity to dNTPs and can work at very low dNTP levels. In addition, growth inhibition of *Uu* was performed with several nucleoside analogs. 5-FdUrd showed to be the most potent inhibitor and 5-FdCyd was at second place. It was shown that 5-FdCyd inhibited *Uu* via its deaminated product, 5-FdUMP. The involvement of *Uu*-TK and the salvage enzymes were further clarified when competition studies showed how addition of dThd or dUrd to the growth media could protect *Uu* from inhibition by 5-FdUrd.

Paper II: Structures of thymidine kinase 1 of human and mycoplasmic origin

In search for inhibitory agents selective for a specific target, such as an enzyme, an available structure of the target is of great help. The structure will provide additional information of the target that cannot be obtained solely through biochemical studies. *Uu*-TK belongs to the TK1 family and there was no known structure in this family of enzymes when the project on *Uu*-TK started. The *Uu*-TK and human TK1 structures were solved in collaboration with Hans Eklund's group (SLU, Uppsala, Sweden) and the purified *Uu*-TK was provided by me and hTK1 from B. Munch-Petersen's lab (Roskilde University, Roskilde, Denmark).

Uu-TK and a C-terminal truncated version of human TK1 (hTK1) were both co-crystallized in complex with dTTP. Both structures were tetramers and each monomer consisted of two domains, an α/β domain and an unexpected small lasso- and zinc-containing domain. The active site was situated between these domains. The α/β domain had a central six-stranded parallel β-sheet with a long helix and a flexible loop on one side and three shorter helices on the other side. The smaller domain had a long lasso loop that covered the substrate binding site and two perpendicular β-strands that were kept in place by a zinc ion. A conserved arginine-tyrosine couple from the two β-strands coordinated the lasso loop. The monomers interacted in two different ways in the tetramer that had a central channel lined with mainly polar and charged residues. One of the interactions was mainly between two long helices forming an antiparallel pair. The other way of interaction was between the two β-sheets that were connected in an antiparallel manner with water molecules in between. In *Uu*-TK the C-terminal helix also interacted with a corresponding helix in an adjacent monomer in the second type of interaction. Of the known structures today the *Uu*-TK structure is the only one that has a visible C-terminal and thereby is the only model for C-terminal subunit interaction. The hTK1 structure is truncated in its C-terminus and several bacterial TKs have a very short C-terminal.

dTTP was bound in the substrate site with the triphosphates bound to the phosphate binding site. The base and deoxyribose of dTTP was more buried into a cleft between the α/β domain and lasso- and zinc-domain whereas the triphosphates were more exposed and bound solely to the α/β domain. The hydrogen bonds between dTTP and the enzyme were almost only main-chain bonds.

The two TK structures were very similar (rmsd 1.3 Å for 155 C_α atoms), despite the low sequence identity (29%). The fold was different from other deoxyribonucleoside kinases (dNKs) as well. The lasso- and zinc-containing domain was not found in any other known protein structure and the α/β domain was also different from the dNK structures and instead similar to enzymes in the RecA-F_1ATPase family. These structural differences between *Uu*-/hTK1 and other dNKs indicate a different evolutionary origin of the TK1 family.

Paper III: Structure of the substrate complex of thymidine kinase from Ureaplasma urealyticum and investigations of possible drug targets for the enzyme

In this paper the phosphorylation of and inhibition by several nucleoside analogs of *Uu*-TK were studied in the light of a new structure of *Uu*-TK in complex with dThd.

A *Uu*-TK structure was solved in complex with dThd and the structural differences, when dThd was bound instead of dTTP, were close to the phosphate binding site, where the conformation of the flexible loop was different. In the complex with dThd the loop was more disordered than in the one with dTTP. This loop was visible in only a few subunits in both complexes, indeed indicating a high flexibility. Glutamate 97 (Glu97) had also moved towards dThd in that complex. Glu97 was hydrogen bonded to the 5'-oxygen in dThd, which means that Glu97 is the catalytic base in the phosphoryl transfer reaction.

Several nucleoside analogs were tested on *Uu*-TK. 5-halogenated analogs were good substrates and had the highest activities. The 5-position on the base tolerated substitutions as large as a cyclopropyl or an ethyl group and the activity was about half of that of dThd. With bulkier modifications the activity decreased and no activity was detected with a bromovinyl group. This steric hindrance correlates well with the hydrophobic pocket the 5-methyl group in dThd normally fits in. Three N3 modified analogs with alkyl substitutions were tested and they all had lower activity than dThd. The hydrogen bond of the N3 nitrogen to the lasso is lost in the analogs. Hence, a tight spacing of the lasso is probably needed for good phosphorylation. The 3'-hydroxyl group was also hydrogen bonded to the lasso. The 3'-analogs with polar substituents could still form hydrogen bonds and retained substantial activity whereas analogs with nonpolar groups showed very low activity. Analogs with 2'-modifications showed the lowest activity of all tested analogs. These results agree well with the tight spacing around both sides of the 2'-position. However, fluorine in the 2'-arabinosyl position was accepted and about two fifths of the activity of dThd was retained. Two of the tested analogs

showed higher relative activity with *Uu*-TK than with hTK1, FCPU (3'-fluoro-5-cyclopropyldUrd) and FLT. Further kinetics showed that both the analogs were less efficient with *Uu*-TK than with hTK1. Some analogs were also chosen for analysis as inhibitors of dThd phosphorylation. More than half of the analogs had had quite low IC_{50} values (the concentration when 50% of the activity of the enzyme is inhibited), but corresponding values for hTK1 were in general lower. Two analogs had higher values with hTK1 than with *Uu*-TK, but they were not very efficiently phosphorylated by *Uu*-TK. The results from this study have provided ideas for further development of selective *Uu*-TK inhibitors, to explore the 5-position, the 3'-position and combinations thereof.

Paper IV: Evaluation of Bacillus anthracis thymidine kinase as a potential target for the development of antibacterial nucleoside analogs

Bacillus anthracis (*B. anthracis*) causes anthrax, which in the inhalation form has fatal consequences. Due to its spores and high infectiousness *B. anthracis* may be a highly potent bioweapon and was lately used as such in association with 9/11 2001 in USA. For those reasons we decided to investigate thymidine kinase in *B. anthracis* (*Ba*-TK) as a target for future development of anti-anthrax agents. Additionally, growth inhibition of *B. anthracis* with selected nucleoside analogs was studied.

Ba-TK was cloned and expressed in an *E. coli* system. The protein was affinity purified in high amounts (ca 25 mg protein/L). Active *Ba*-TK was a dimer as determined by gel filtration. Similar to *Uu*-TK and in contrast to hTK1, ATP had no effect on subunit interactions of *Ba*-TK. *Ba*-TK, phosphorylated only dThd and dUrd. Substrate inhibition was observed with dThd (>15 μM) but not with dUrd. Furthermore, *Ba*-TK used all nucleoside triphosphates as donors with a clear preference for guanosine. Surprisingly, dTTP showed very low but still some activity with *Ba*-TK in contrast to *Uu*-TK and hTK1. Several nucleoside analogs were tested and compounds with halogen substituents at the 5-position were the best substrates. With bulkier modifications the activity decreased, indicating a size sensitivity at this position. The 3'-analogs FLT and AZT also showed good activity but d4T did not. These results signify a prerequisite for binding interactions in both the 3'- and 2'-position to retain activity of the enzyme. The tested N3 analogs with alkyl substituents showed similar activity with *Ba*-TK as with *Uu*-TK suggesting that hydrogen bonds with the N3 nitrogen are lost in the analogs. The substrate and analog specificity of *Ba*-TK resembles the specificities of *Uu*-TK and hTK1 indicating that *Ba*-TK has the same overall structure as the two other enzymes and belongs to the TK1 family.

A number of nucleoside analogs and other compounds of pharmacological importance were chosen to study their inhibitory effect on *B. anthracis* growth. Three compounds showed an ED_{50} value (effective dose) less than 200 μM; 5-FU, 5-FdUrd and N5-2OH. There was no obvious correlation between the growth inhibitory effect and substrate specificity of *Ba*-TK of these analogs. This implies that there could be other enzymes or targets involved in toxicity of these compounds.

Paper V: Molecular characterization of thymidylate kinase from Bacillus anthracis

Thymidylate kinase (TMPK) is at the junction of the *de novo* and the salvage pathway in dTTP synthesis, which makes it an interesting and potent target for drug design. Thus, we decided to characterize TMPK from *B. anthracis* (*Ba*-TMPK) with focus on its substrate specificity. The results may be used in development of agents against *B. anthracis*.

Ba-TMPK was cloned and expressed in an *E. coli* system. The protein was affinity purified to the very high amount of 180 mg/L. Gel filtration revealed that *Ba*-TMPK was a dimer, as all other known TMPKs. Regarding phosphate donors, *Ba*-TMPK used ATP and dATP as efficient donors but had low affinity towards other nucleoside triphosphates. *Ba*-TMPK recognized dTMP as substrate and phosphorylated dUMP with a tenfold lower activity. Other phosphate acceptors were very poor substrates for *Ba*-TMPK. Hence, *Ba*-TMPK is strictly thymidylate specific. This low affinity for dUMP is found in other TMPKs as well, which may imply a safety mechanism of the organism to prevent eventual excess of dUDP/dUTP, causing replication mistakes. Some dTMP analogs were also tested and FMAUMP gave the best response (with three times lower efficiency compared to dTMP). 5-FdUMP and d4TMP were poor substrates and no activity could be detected with AZTMP. These results show the sensitivity of the enzyme for 5-position substitutions but also how sensitive *Ba*-TMPK is for 3'-modifications. Fluorine as substituent in the 2'-arabinosyl position is on the other hand tolerated. The nucleoside forms of the above-mentioned analogs were tested as inhibitors of dTMP phosphorylation and the same pattern was observed, with FMAU being the best inhibitor. The inhibition pattern implies a surprisingly minor importance of the 5'-phosphate compared to the nucleoside moiety in substrate binding affinity. Several nucleoside analogs with rather bulky 5'-modifications designed as non-substrate inhibitors were tested on *Ba*-TMPK as well. A few of the analogs inhibited the enzyme up to 30%. These analogs and FMAU(MP) are the most promising leads for future research on agents against *B. anthracis*.

Concluding remarks and future perspectives

Three enzymes in the thymidylate synthesis have been studied in this thesis; thymidine kinase and thymidylate kinase from *Bacillus anhracis* (*Ba*-TK and *Ba*-TMPK) and thymidine kinase from *Ureaplasma urealyticum* (*Uu*-TK). Additionally, the structure of *Uu*-TK has been solved together with co-workers.

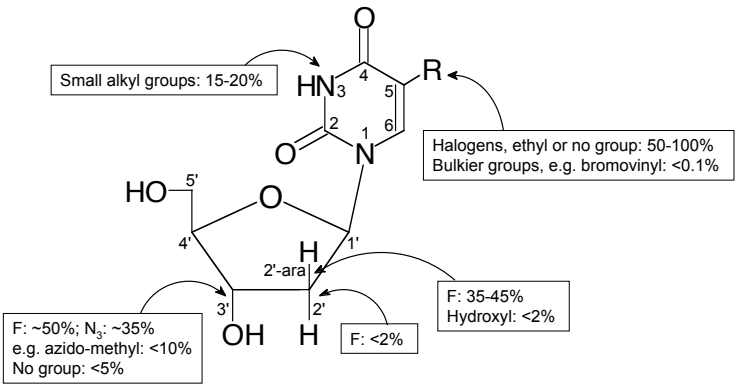
In this thesis we have shown that the TK1 family differs both enzymatically and structurally from other dNKs - having a more narrow substrate specificity and a completely different 3D structure. The overall structure of enzymes in the TK1 family is similar to the RecA-F_1ATPase enzyme family, but TK1 enzymes also have a unique domain baptized to the lasso- and zinc-domain. The substrate specificities of the TK1 enzymes resemble one another and many of the interactions between the substrate and the enzyme are to the backbone of the protein. There are, however, minor differences in the active site between TK1 enzymes. Furthermore, it takes a whole enzyme to do the reaction. The major part of the reaction outcome is of course due to the active site but other parts of the protein can be important just as well. In the present investigations we have taken the first step when looking for potential antibacterial agents and found a number of leads. The next step would be further exploration of those leads and investigations of the active site in the search for specific inhibitors.

Despite the similarities in substrate specificity between TK1 enzymes they have different phosphorylation efficiencies. This feature has to be taken into account in drug design. *In vitro* studies of TK1 enzymes are mainly done with recombinant proteins, as in the case of *Uu*-TK and *Ba*-TK. The phosphorylation efficiency, the abundance and expression of the enzymes are not necessarily the same *in vivo*. Hence, there are many aspects that are needed to be considered. One more is the multiple steps involved in activating nucleoside analogs from their prodrug form to their active form, where dNKs are responsible for the first step. The present studies with *Ba*-TK and *Ba*-TMPK have revealed that the enzymes have similar substrate preferences with leads in the 2'-arabinosyl position and possibly in the 5-position on the base (figure 8). The phosphorylated products from the TK reaction will then be further phosphorylated by the TMPK avoiding accumulation. *Ba*-TMPK is also the site where the salvage and the *de novo* synthesis meet in nucleotide synthesis and therefore is a potential target for non-substrate analogs. Blocking TMPK will in turn stop both pathways to dTTP synthesis that will eventually lead to cell death.

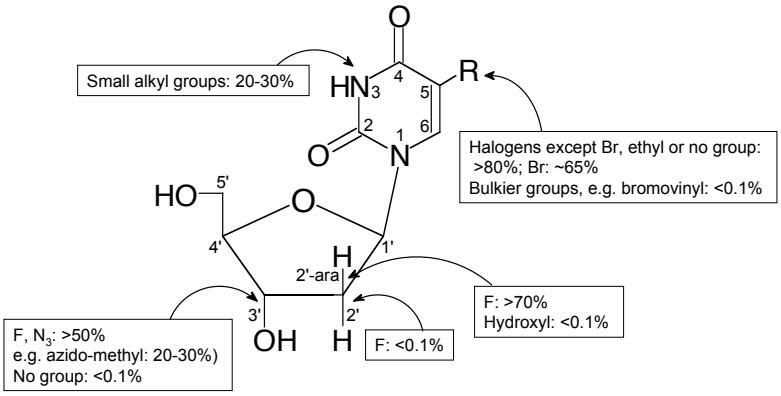
Figure 8 summarizes the substrate analog specificity of *Uu*-TK, *Ba*-TK and *Ba*-TMPK, respectively. Except the position preferences mentioned above *Uu*-TK and *Ba*-TK also tolerate substitutions at the 3'-position. Additionally, *Uu*-TK accepts modifications at the 2'-arabinosyl position. These substitution differences are interesting and should be investigated further. They will also be helpful in finding and developing new substrate analogs and inhibitors for the three enzymes in question.

The results of the present studies have given a deeper structural and functional understanding of enzymes in the thymidylate synthesis. The acquired knowledge will aid in defining the differences and similarities in thymidylate synthesis and be of value in future drug design.

(A)



(B)



(C)

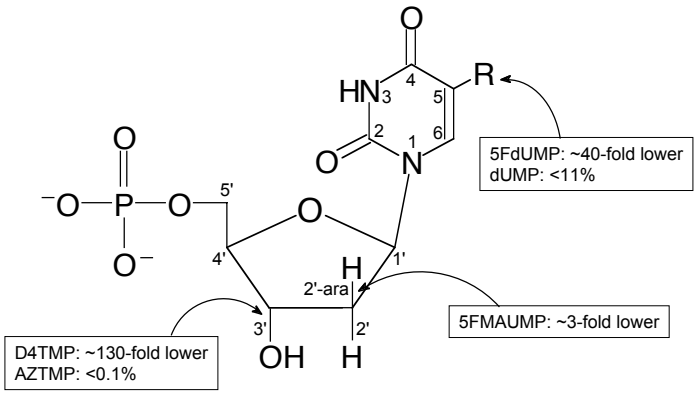


Figure 8. (A) *Uu*-TK (B) *Ba*-TK and (C) *Ba*-TMPK activity of substrate analogs. The arrows indicate the modifications and the percentage values are the enzyme activity with 100 μM substrate analog as compared to that with dTMP (= 100%). In (C) the substrate analog concentration was 1 mM and the three other values represent the enzyme efficiency (k_{cat}/K_m) as compared to that with dTMP.

Conclusions

- *Uu*-TK and *Ba*-TK are strictly pyrimidine specific and regarding their analog specificity: 5-Halogenated analogs give the highest activity. Modifications at the 3-position on the base give moderate activities while 3'-analogs in general have a higher activity. 2'-analogs are essentially not substrates but a fluorine substitution at the 2'-arabinosyl position is accepted.

- *Uu*-TK and *Ba*-TK can use all nucleoside triphosphates as phosphate donors except dTTP, which is a very poor phosphate donor for *Ba*-TK and a feedback inhibitor for *Uu*-TK.

- The *Uu*-TK structure, i.e. a TK1 enzyme structure, is unique, containing a long loop and a structural zinc ion - the lasso domain. The core structures of TK1 enzymes resemble an ATPase family and are not related to the other dNKs.

- *Ba*-TMPK is a thymidylate specific kinase and uses ATP or dATP as phosphate donors.

- FMAUMP is a good substrate for *Ba*-TMPK and its nucleoside form is a potent inhibitor. FMAU(MP) may thus serve as a lead for future design of antibiotics against *B. anthracis*.

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In this thesis, thymidine and thymidylate kinases from *Bacillus anthracis* (*Ba*-TK and *Ba*-TMPK) and TK from *Ureaplasma urealyticum* (*Uu*-TK) are studied with respect to substrate specificity and 3D structure. The TKs belong enzymatically and structurally to the TK1 family and the activities of nucleoside analogs with the TKs are in the order 5- ≥3'- and 2'-ara- >N3- >2'-modifications. *Ba*-TMPK is thymidylate specific and among analogs with 5-, 2'- or 3'-modifications a 2'-ara-analog gives the highest activity.

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