Structural Studies of Salvage Enzymes in Nucleotide Biosynthesis

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Abstract

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There are two routes to produce deoxyribonucleoside triphosphates (dNTPs) precursors for DNA synthesis, the *de novo* and the salvage pathways. Deoxyribonucleoside kinases (dNKs) perform the initial phosphorylation of deoxyribonucleosides (dNs). Furthermore, they can act as activators for several medically important nucleoside analogs (NAs) for treatment against cancer or viral infections. Several disorders are characterized by mutations in enzymes involved in the nucleotide biosynthesis, such as Lesch-Nyhan disease that is linked to hypoxanthine guanine phosphoribosyltransferase (HPRT).

In this thesis, the structures of human thymidine kinase 1 (TK1), a mycoplasmic deoxyadenosine kinase (*Mm*-dAK), and phosphoribosyltransferase domain containing 1 (PRTFDC1) are presented. Furthermore, a structural investigation of *Drosophila melanogaster* dNK (*Dm*-dNK) N64D mutant was carried out. The obtained structural information reveals the basis for substrate specificity for TKs and the bacterial dAKs. The TK1 revealed a structure different from other known dNK structures, containing an α/β domain similar to the RecA-F_1ATPase family, and a lasso-like domain stabilized by a structural zinc. The *Mm*-dAK structure was similar to its human counterparts, but with some alterations in the proximity of the active site. Furthermore several residues important for substrate specificity were identified. The crystal structure of PRTFDC1 was structurally very similar to its homolog HPRT. PRTFDC1 was classified as having an unknown function and with structural and biochemical data we showed that PRTFDC1 has some phosphoribosyltransferase activity. A changed behavior of the *Dm*-dNK N64D mutant was previously observed. This mutant displayed an increased sensitivity towards NAs and a decreased feedback inhibition. Complexes with substrate and feedback inhibitor provided an explanation for the changed behavior of the mutant.

The structural data presented here, provide a foundation for substrate specificity for dNKs. The information of differences between human and bacterial enzymes will be of importance for the design of new anti-bacterial agents. Mutational studies to improve desired properties of an enzyme are an important issue in suicide gene/chemotherapy. Although we have found a potential function of PRTFDC1 there are still a lot of questions concerning its biological role to answer.

Keywords: thymidine kinase 1, deoxyribonucleoside kinase, deoxyadenosine kinase, mycoplasma, phosphoribosyltransferase domain containing 1, *Drosophila melanogaster*, crystal structure

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To my family

Contents

Appendix

Papers I-IV

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

I. *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 10*, 17970-5.

II. Welin, M., Skovgaard, T., Knecht, W., Zhu, C., Berenstein, D., Munch-Petersen, B., Piskur, J. & Eklund, H. 2005. Structural basis for the changed substrate specificity of *Drosophila melanogaster* deoxyribonucleoside kinase mutant N64D. *Febs J 272*, 3733-42.

III. Welin, M., Wang, L., Eriksson, S. & Eklund, H. 2007. Structure-function analysis of a bacterial deoxyadenosine kinase reveals the basis for substrate specificity. *J Mol Biol 366*, 1615-23.

IV. Welin, M., Stenmark, P., Wang, L., Flodin, S., Nyman, T., Kotenyova, T., Johansson, I., Eriksson, S., Eklund, H. & Nordlund, P. Structural and functional investigation of the human phosphoribosyltransferase containing domain 1 - possible implications for the Lesch-Nyhan disease. (Manuscript).

*Shared first authorship

Abbreviations

Enzymes:

RNR	ribonucleotide reductase
dNK	deoxyribonucleoside kinases
TK1	human thymidine kinase
dCK	human deoxycytidine kinase
TK2	human thymidine kinase 2
dGK	human deoxyguanosine kinase
Dm-dNK	*Drosophila melanogaster* dNK
Uu-TK	*Ureaplasma urealyticum* thymidine kinase
Mm-dAK	*Mycoplasma mycoides* subsp. *mycoides* SC deoxyadenosine kinase
HSV1-TK	Herpes simplex virus type 1 thymidine kinase
HPRT	hypoxanthine guanine phosphoribosyltransferase
APRT	adenine phosphoribosyltransferase
PRTFDC1	phosphoribosyltransferase domain containing 1

Nucleosides/nucleotides

dNTP	deoxyribonucleoside triphophates
dN	deoxyribonucleoside
NMP	ribonucleoside monophosphates
dT	deoxythymidine
dU	deoxyuridine
dC	deoxycytidine
dG	deoxyguanosine
-MP	monophosphate
-DP	diphosphate
-TP	triphosphate

Nucleoside analogs

NA	nucleoside analog
dFdC	2´,2´-difluoro-2´-deoxycytidine
5FdU	5-fluoro-2´-deoxyuridine
AZT	3´azido-2´,3´dideoxythymidine
ddC	2´,3´-dideoxycytidine
araG	9-β-D-arabinofuranosyl guanine
GCV	9-(1,3-dihydroxy-2-propoxymethyl)-guanine

1. Introduction

1.1. Production of DNA precursors

Each of the four deoxyribonucleoside triphosphates (dNTPs) is required for a cell to replicate and repair DNA. The constituents building up a deoxyribonucleoside (dN) are a purine (adenine or guanine) or pyrimidine base (thymine and cytosine) and a deoxyribose (Figure 1). In a cell there are two routes to produce dNTPs, the *de novo* and the salvage pathways.

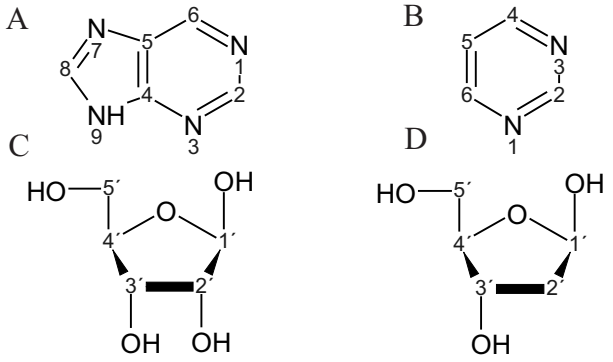

Figure 1. Illustrations of (A) purine, (B) pyrimidine, (C) ribose and (D) deoxyribose.

1.1.1. De novo pathway

In the *de novo* pathway purine and pyrimidine (d)NTPs are produced. There are two separate ways to make purine and pyrimidine nucleotides. Purine nucleoside monophosphates (NMPs) are synthesized from several smaller compounds in numerous reactions performed by several different enzymes. Regarding pyrimidine synthesis, a pyrimidine base is first composed through several reactions. The bases are then used to form NMPs. The NMPs are substrates for nucleoside monophosphate kinases ((d)NMPKs) that add a second phosphoryl group to the NMPs. The formed nucleoside diphosphates (NDPs) are either phosphorylated to nucleoside triphosphates (NTPs) by nucleoside diphosphate kinases (NDPKs) for RNA synthesis or reduced to deoxyribonucleoside diphosphates (dNDPs) by ribonucleotide reductase (RNR) (Thelander & Reichard, 1979). A third phosphoryl group is then added to form dNTPs, precursors of DNA. To produce deoxythymidine monophosphate (dTMP) a different route is taken, where deoxyuridine monophosphates (dUMPs) are methylated performed by the enzyme thymidylate synthase (Montfort & Weichsel, 1997).

1.1.2. Salvage pathway

The second route, the salvage pathway recycles nucleobases and dNs from nutrients and degraded DNA (Figure 2). The dNs are transported into the cells through nucleoside transporter proteins (Plagemann, Wohlhueter & Woffendin, 1988). The first step in the salvage pathway, which leads towards production of

dNTPs, is to trap the dNs in the cell. This is accomplished by the transfer of a phosphoryl group from NTP to a dN by deoxyribonucleoside kinases (dNKs). The charge of the dNMP traps the molecule inside the cell and thus transportation out of the cell is hindered. Now the deoxyribonucleoside monophosphates (dNMPs) can be further phosphorylated to its respective dNTP by (d)NMPKs and NDPKs. In addition there is an ongoing recycling of nucleobases catalyzed by enzymes, specifically phosphoribosyltransferases (PRTs). Degraded nucleobases are used to form NMPs for either RNA or DNA synthesis (Sinha & Smith, 2001).

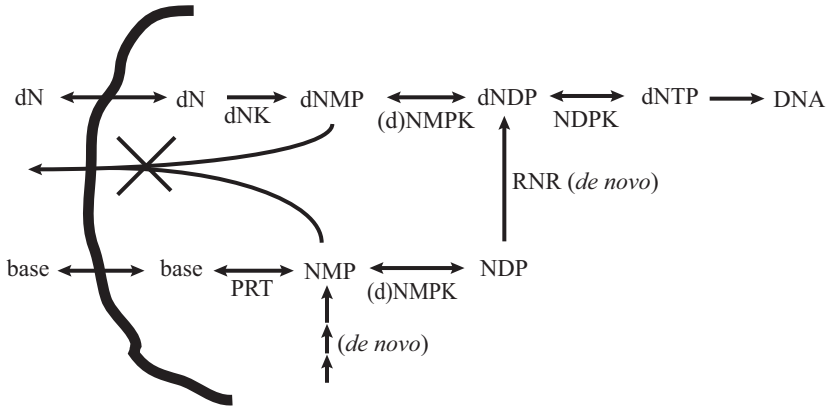

Figure 2. Simplified scheme of the production of precursors for DNA synthesis, dNTPs.

1.2. Deoxyribonucleoside kinases (dNKs)

dNKs are enzymes catalyzing the first step in the salvage pathway. They perform the transfer of a phosphoryl group from a NTP to dN. The dNKs are found in a broad range of species in different numbers.

1.2.1. Human dNKs

There are four different dNKs in a mammalian cell. Two dNKs are situated in the cytosol, thymidine kinase 1 (TK1, E.C. number: 2.7.1.21) and deoxycytidine kinase (dCK, E.C. number: 2.7.1.74) and two in the mitochondria, thymidine kinase 2 (TK2, E.C. number: 2.7.1.21) and deoxyguanosine kinase (dGK, E.C. number: 2.7.1.113) (Arnér & Eriksson, 1995; Eriksson *et al.*, 2002). These enzymes have complementary substrate specificity and are able to phosphorylate all of the natural substrates (Table 1). TK1 has a stricter specificity than the other dNKs, and can only phosphorylate deoxythymidine (dT) and deoxyuridine (dU). dCK is responsible for the phosphorylation of the remaining substrates in the cytosol, deoxycytidine (dC), deoxyadenosine (dA) and deoxyguanosine (dG). In the mitochondria there is one pyrimidine specific enzyme, TK2 that can phosphorylate dT, dU and dC and a more purine specific, dGK that utilizes dA and dG and to some extent dC (Arnér & Eriksson, 1995; Eriksson*, et al.*, 2002). Of the human dNKs, TK1 will be discussed in greater detail in paper I.

	Location	dT	dU	dC	dG	dA
TK1	cytosol	+	+	-	-	-
dCK	cytosol	-	-	+	+	+
TK2	mitochondria	+	+	+	-	-
dGK	mitochondria	-	-	(+)	+	+

Table 1. Substrate specificity with natural substrates of the human dNKs. + indicates activity, - indicates no activity, (+) indicates some activity, activities are taken from (Eriksson*, et al.*, 2002).

1.2.2. Multisubstrate dNK

Many organisms have been shown to have multiple dNKs with complementary substrate specificity. However in insects there is only one dNK with broader substrate specificity able to phosphorylate all natural dNs. The first multisubstrate dNK to be discovered and cloned was *Drosophila melanogaster* dNK (*Dm*-dNK, E.C. number: 2.7.1.145) (Johansson *et al.*, 1999; Munch-Petersen *et al.*, 2000; Munch-Petersen, Piskur & Søndergaard, 1998). Sequencing of the entire *D. melanogaster* genome revealed that *Dm*-dNK indeed was the only existing dNK (Adams *et al.*, 2000). At the present time several other insect dNKs have been identified, such as *Anopheles gambiae* and *Bombyx mori* dNK, that also can utilize all dNs (Knecht *et al.*, 2002a; Knecht *et al.*, 2003). *Dm*-dNK will be discussed in greater detail in paper II.

1.2.3. Bacterial dNKs

The first bacterial dNK to be discovered and characterized was TK from *Escherichia coli* (Okazaki & Kornberg, 1964a; Okazaki & Kornberg, 1964b). Recently several TKs from gram positive bacteria have been characterized e.g. *Ureaplasma urealyticum* (*parvum*) (*Uu*-TK) and *Bacillus anthracis* (*Ba*-TK) (Carnrot *et al.*, 2003; Carnrot *et al.*, 2006). Other dNKs to be characterized were the deoxyadenosine/deoxycytidine kinase from *Bacillus subtilis,* which shows specificity towards dA and dC (Møllgaard, 1980), and a deoxyguanosine kinase (Andersen & Neuhard, 2001). In *Lactobacillus acidophilus* R-26 two heterodimeric kinases were discovered; one dAK-dGK and one dAK-dCK (Ives & Ikeda, 1998; Ma *et al.*, 1996). Recently a deoxyadenosine kinase from *Mycoplasma mycoides* subsp. *mycoides* SC (*Mm*-dAK) has been characterized (Pol & Wang, 2006; Wang *et al.*, 2001) and this enzyme will be described in detail in paper III.

1.2.4. Viral TKs

Herpes simplex virus type 1 TK (HSV1-TK) is a homodimer with a molecular weight of ca 80 kDa (Chen & Prusoff, 1978), while Vaccinia virus TK (VV-TK) is a tetramer of the same size (Black & Hruby, 1990). These dNKs are referred to as viral TKs since they have preferences towards dT (Gentry, 1992). The VV-TK is similar to eukaryotic and bacterial TKs but with a shorter C-terminus. HSV1-TK

has a broad specificity and can phosphorylate both dT and dC. In addition it has thymidylate activity (Chen & Prusoff, 1978; Jamieson & Subak-Sharpe, 1974). VV-TK has a narrower substrate specificity that is similar to human TK1 (Solaroli *et al.*, 2006b).

1.3. Two families of dNKs

The first crystal structure of a dNK to be determined was HSV1-TK (Brown *et al.*, 1995; Wild *et al.*, 1995), and at the present time structural data is readily available. Based on the different folds of the dNKs, the structures can be divided into two families, a dCK/dGK family (including some viral TKs, such as HSV1-TK) and a TK1 family. Nucleotide binding proteins usually possess a phosphate-binding loop (P-loop), the only motif shared by the two families. Among the four human dNKs the crystal structures of dGK, dCK and TK1 have been determined (Birringer *et al.*, 2005; Johansson *et al.*, 2001; Sabini *et al.*, 2003; Welin *et al.*, 2004).

1.3.1. dCK/dGK family

The dGK and dCK structures are both structurally and sequentially similar. Superimposition with the HSV1-TK revealed structural similarity despite low sequence identity. Several conserved residues between the structures were identified: (i), a glutamine which is hydrogen bonded to the substrate base, (ii), a Glu-Tyr couple that is hydrogen bonded to the 3´-hydroxyl group of the deoxyribose moiety and (iii), a Glu-Arg couple that is hydrogen bonded to the 5´OH group of the substrate (Johansson*, et al.*, 2001; Wild*, et al.*, 1995; Wild *et al.*, 1997). It was suggested by Wild, *et al.* (1997) that the glutamic acid in the Glu-Arg couple was involved in catalysis. The glutamic acid acts as a base and deprotonates the 5´OH of the substrate, allowing the substrate to make a nucleophilic attack on the γ-phosphate of the phosphate donor (Wild*, et al.*, 1997) (Figure 3). Magnesium is required for this reaction and several arginines in proximity help to stabilize the transition state. A mutational study of the Glu52 in *Dm*-dNK revealed total abolishment of the activity when mutated to a glutamine (Egeblad-Welin *et al.*, 2007). The glutamic acid is found in proximity to 5´OH of the deoxyribose moiety of all known dNK structures, suggesting a general mechanism for all dNKs. The structure of the multisubstrate dNK, *Dm*-dNK revealed a broader dN binding site and thus provides an explanation for the ability of *Dm*-dNK to phosphorylate all four natural substrates (Eriksson*, et al.*, 2002; Johansson*, et al.*, 2001). Other recently solved structures belonging to this family are the Varicella zoster virus TK (VZV-TK) (Bird *et al.*, 2003) and the first bacterial enzyme belonging to this family *Mm*-dAK (Welin *et al.*, 2007).

1.3.2. TK1 family

Recent studies have provided significant data concerning the structure of TKs. The sequences within the TK1 family are well conserved, and the structures of the human TK1, *Uu*-TK, *Clostridium acetobutylicum* TK (*Ca*-TK), VV-TK, *Ba*-TK and a TK from *Bacillus cereus* showed that they are structurally very similar (Birringer*, et al.*, 2005; El Omari *et al.*, 2006; Kosinska *et al.*, 2007; Kuzin *et al.*, 2004; Welin*, et al.*, 2004). The TK structures revealed a core domain consisting of

a six stranded parallel β-sheet sandwiched between several α-helices, and thus resemble the ATP binding domain of the RecA-F_1ATPase family (Sawaya *et al.*, 1999). A second domain, with a lasso like loop and a structural zinc was also revealed in the TK1 structure (Welin*, et al.*, 2004).

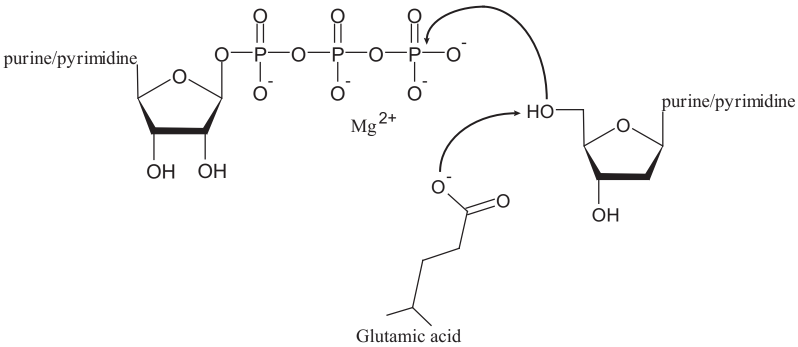

Figure 3. General mechanism for dNKs, (the figure has been modified from (Eriksson *et al.*, 2002)).

1.4. Feedback inhibition

Initial studies showed that several dNKs could be inhibited by their distal end product. In the early sixties it was suggested for TK1 that dTTP overlaps both the acceptor and the donor site (Ives, Morse & Potter, 1963). The crystal structures of *Dm*-dNK with substrate and feedback inhibitor bound confirmed this type of overlapping binding mode (Eriksson*, et al.*, 2002; Mikkelsen *et al.*, 2003). The end product of the best substrate for a dNK has been shown to be the most potent feedback inhibitor.

1.5. Medical interests

1.5.1. Nucleoside analogs

Nucleoside analogs (NA) or pro-drugs are non-toxic until activation through phosphorylation by kinases. The NAs are transported into a cell using the same transporter system as natural dNs (Plagemann, Wohlhueter & Woffendin, 1988). Inside the cell the NAs are phosphorylated, a crucial step for keeping the NA inside the cell. This first activation step is carried out by dNKs which phosphorylate NAs into a NA-monophosphate (NAMP). The NAMPs are then phosphorylated in two steps to NA-triphosphate by NMPK and NDPK (Van Rompay, Johansson & Karlsson, 2000). The activated form of an NA can act as a terminator of DNA elongation, interfere with reverse transcriptase or cellular DNA polymerases, and induce apoptosis (Klopfer *et al.*, 2004; Macchi & Mastino, 2002). Furthermore, some NAs can inhibit other enzymes in the nucleotide biosynthesis pathway simultaneously, like 2´,2´-difluoro-2´-deoxycytidine (dFdC) inactivates RNR in its diphosphate form (Ostruszka & Shewach, 2003). TK1 phosphorylates 5-fluoro-2´-deoxyuridine (5FdU) to its active form, 5FdU-monophosphate, which inhibits thymidylate synthase (Santi & McHenry, 1972).

1.5.2. Suicide gene/chemotherapy

The basic idea of suicide gene/chemotherapy is to transfer a gene that can activate a specific NA into cancer cells, hence killing the cancer cells by administration of NAs (Niculescu-Duvaz & Springer, 2005). This is accomplished through first delivering the gene of interest, which encodes the enzyme, into the cancer cells via a vector, e.g. adenoviruses. Subsequent expression of the gene inside the cancer cells followed by the administration of the NA ultimately kills the cancer cells. This has been implemented using HSV1-TK and 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (GCV), an NA not phosphorylated by the human dNKs (Moolten, 1986). By the bystander effect neighboring cells are killed by the transfer of the activated NAs by cell-cell contacts (Mesnil & Yamasaki, 2000). By fusing the genes of HSV1-TK and guanosine monophosphate kinase (GMPK) a lower IC_{50} was observed compared to the HSV1-TK alone (Willmon, Krabbenhoft & Black, 2006). A clinical trial using HSV1-TK gene in a nonreplicative adenoviral vector in combination with GCV, towards malignant mesothelioma increased the survival time for some patients (Sterman *et al.*, 2005).

1.6. Purine phosphoribosyltransferases

Purine phosphoribosyltransferases are salvage enzymes recycling purine nucleobases hypoxanthine (Hx), guanine (G) and adenine (A) to form the respective NMPs using α-D-5-phosphoribosyl 1-pyrophosphate (PRPP) and Mg^{2+} in a reversible reaction (Figure 4). In human cells there are two well described enzymes carrying out these reactions, hypoxanthine guanine phosphoribosyltransferase (HPRT, E.C. number: 2.4.2.8) and adenine phosphoribosyltransferase (APRT, E.C. number: 2.4.2.7). The HPRT has been intensively studied for mainly two reasons. First, most protozoan parasites lack the *de novo* pathway of purine nucleotides and have to depend on their salvage enzymes. Parasitic HPRTs have thereby become a potential target for the development of anti-parasitic drugs (Wang, 1984). Second, mutations in human HPRT and Lesch-Nyhan syndrome are linked, as described below. HPRT and its homolog phosphoribosyltransferase domain containing 1 (PRTFDC1) will be discussed in more detail in paper IV.

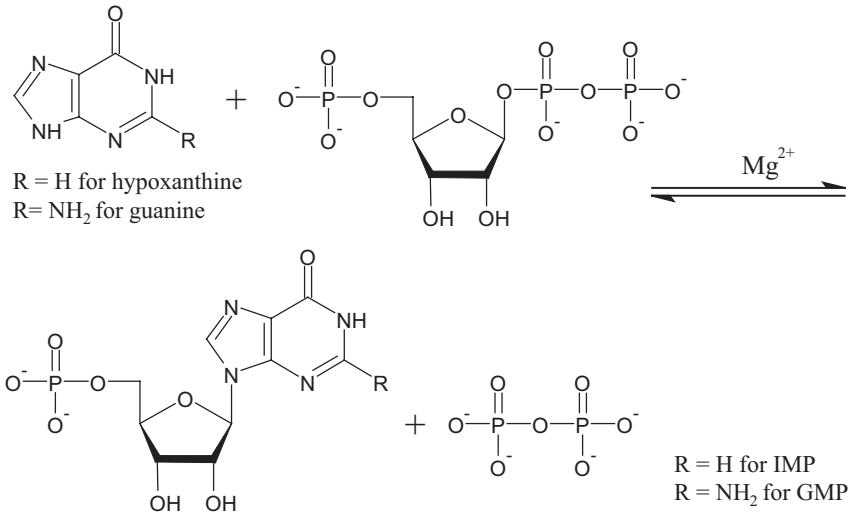

Figure 4. The reaction catalyzed by HPRT.

1.7. Disorders

Several disorders are characterized by mutations or lack of enzymes in the nucleotide biosynthesis process. Mitochondrial DNA depletion syndrome (MDS) is characterized by a quantitative defect of mitochondrial DNA (Moraes *et al.*, 1991). Mutations in dGK and TK2 have been linked to MDS, resulting in symptoms such as myopathy and liver failure (Mandel *et al.*, 2001; Moraes*, et al.*, 1991; Saada *et al.*, 2001). Mutations in the HPRT gene that lead to an almost inactive enzyme, giving rise to a disorder called Lesch-Nyhan disease. Patients having the disorder display symptoms such as retarded motor development and self injurious behavior (Nyhan, 2005). This disease was first described by Michael Lesch and William Nyhan in 1964 (Lesch & Nyhan, 1964). Some mutations in HPRT lead to partial activity of the enzyme and can cause gout arthritis (Jinnah *et al.*, 2000).

2.1. Human thymidine kinase 1, TK1 (Paper I)

2.2. Aim of study

At the time, though several crystal structures of enzymes belonging to the dCK/dGK family have been determined, the structure of a TK1 family enzyme had not been solved, despite its biological importance and its role as an activator of several pro-drugs. Low sequence identity indicated a structure different from the dCK/dGK family. Our aim was to determine TK structures of both human and mycoplasmic origin, and to study potential differences for the design of new anti-microbial agents targeting the bacterial TKs. A further aim of our study was to examine the human TK1 acceptor site and determine how it might be exploited for the development of new pro-drugs.

2.3. Background

More than twenty years ago the first eukaryotic TK gene, the human TK, was cloned and subsequently studied (Bradshaw, 1983; Bradshaw & Deininger, 1984). The gene coding for TK1 is located on chromosome 17 (McDougall, Kucherlapati & Ruddle, 1973; Miller *et al.*, 1971). The human TK1 is a cytosolic enzyme consisting of 234 amino acids and a subunit weight of 24 kDa (Bradshaw & Deininger, 1984; Sherley & Kelly, 1988a). The first published TK1 sequence contained a Met in position 106 though Berenstein, *et al.* (2000) later showed that the correct residue at this position should be a Val (Berenstein *et al.*, 2000; Bradshaw & Deininger, 1984).

TK1 is a cell-cycle regulated enzyme found in dividing cells. The levels of the TK1 enzyme start to increase in late G1 cells, having their peak in the S phase and then disappearing during mitosis (Bello, 1974; Sherley & Kelly, 1988b). While deletion of forty amino acids of the C-terminus of TK1 totally diminishes the cell cycle regulation but retains activity of TK1, deleting only ten residues of the carboxyl terminus does not affect the cell cycle regulation. This suggests that amino acids within the thirty residues of the C-terminus are involved in regulating the cell cycle (Kauffman & Kelly, 1991). Later work revealed that a sequence containing three residues, Lys, Glu and Asn (KEN) was identified in Cdc20, which is a substrate for degradation by the Cdh1-APC complex (Pfleger & Kirschner, 2000). The same KEN sequence was found in the C-terminus of TK1 and mutational studies of this sequence confirmed its necessity for degradation by the APC/C-Cdh1-mediated proteolysis (Ke & Chang, 2004). It has been demonstrated that TK1 gets greatly phosphorylated during the G2 and M phase and shows a decreased affinity towards dT prior to degradation (Chang, Huang & Chi, 1998; Ke *et al.*, 2003). Dietary zinc deficits lead to lower TK activity in rat and sheep fetuses. These findings led to the idea that TK1 was a Zn^{2+} dependent enzyme (Dreosti & Hurley, 1975; Duncan & Hurley, 1978), but this was not confirmed until the first structures of TKs were solved (Welin*, et al.*, 2004). Concerning quaternary structure, both dimeric and tetrameric forms of TK1 have

been observed and are dependent on the presence of ATP. In the absence of ATP TK1 displays a lower affinity towards dT while when incubated with ATP an enzyme with higher affinity for dT is observed (Munch-Petersen, Tyrsted & Cloos, 1993). A recent investigation showed that the wild type TK1 behaved as a tetramer whereas the N and C-termini truncated TK1 behaved as a dimer, independent of incubation with dT and ATP (Birringer *et al.*, 2006).

2.3.1. Substrate specificity

TKs have the most narrow substrate specificity among the dNKs and are only able to phosphorylate dT and dU among the natural substrates (Munch-Petersen *et al.*, 1991). Early studies showed that halogen substitutions at the C5 position of thymine were good substrates for *Ec*-TK, as well as for TK1 (Eriksson *et al.*, 1991; Okazaki & Kornberg, 1964a). TK1 can activate several important pro-drugs for medical use, e.g. the anti HIV drug, 3´azido-2´,3´dideoxythymidine (AZT) and the anti cancer agent, 5FdU (Eriksson*, et al.*, 1991; Furman *et al.*, 1986). Large modifications at the N3 position also work to some extent as phosphate acceptors (Al-Madhoun *et al.*, 2002; Lunato *et al.*, 1999). In summary, modifications at the N3 and C5 positions of the base and at the 3´ position are effective substitution sites.

2.3.2. Medical interests

Since TK1 is expressed in proliferating cells it can be used as a marker in clinical medicine (Gronowitz *et al.*, 1984). Cancer cells rapidly divide making TK1 a marker for different cancer diseases (Li *et al.*, 2005; Zhang *et al.*, 2006). Antibodies that target the C-terminus of TK1 have been designed and may serve as a good diagnostic marker in the detection of abnormal amounts of TK1 in serum (He *et al.*, 1996; He *et al.*, 2000) TK1 can act as an activator of several important NAs, which makes it a target for the design of new NAs or the redesign of old ones. To develop inhibitors targeting bacterial TKs but not the human TK1 would be of interest, especially for bacteria developing multi resistance towards antibiotics.

2.4. Solving the TK1 structure using SIRAS

2.4.1. Crystallization

The TK1 used for crystallization was a truncated version of the protein lacking the last 41 amino acids. To stabilize the protein during the crystallization process it was co-crystallized with its feedback inhibitor, dTTP. Earlier work showed that dTTP stabilizes *Dm*-dNK for crystallization (Mikkelsen*, et al.*, 2003). Crystals were obtained with hanging drops using the vapor diffusion method. Protein crystals grew typically in 6-12 hours as plates and rods to a size of 80x60x60 μm^3 and 220x60x60 μm^3 respectively. A native data set to 2.4 Å resolution was collected at the ESRF.

2.4.2. Heavy atom soaking

Since no similar structures were available at the time, other methods than

molecular replacement had to be considered. To solve the structure, several heavy atoms were soaked into the crystals. By varying the concentrations and the soaking times of the heavy atom solutions a successful combination, 10 mM $KAu(CN)_2$ for 40 min, was found. To remove unspecific bound heavy atoms, 30 s of back-soaking were carried out. A full data set (gold data) was collected at the peak (λ = 1.04 Å). The native data collected and the gold data were non-isomorphous and thus the structure could not be solved. We also collected a data set where the crystal had been soaked in 10 mM lead acetate for 40 min. This data set turned out to be isomorphous to the gold data and could be used for structure determination.

2.4.3. Structure determination

Single isomorphous replacement with anomalous scattering (SIRAS) was applied for structure determination. Using SHELXC/D (Bricogne, 1997; Schneider & Sheldrick, 2002) twelve heavy atom sites could be located. During refinement of these heavy atom sites two new sites were added, so a total of fourteen sites were used in AutoSHARP (Bricogne, 1997; Bricogne *et al.*, 2003). Initial maps were greatly improved with averaging using eight fold non-crystallographic symmetry (NCS) in DM, CCP4 (CCP4, 1994). To match the amino acid sequence into the electron density, the known motif, the P-loop was used. Earlier studies had shown that the feedback inhibitor was bound to the P-loop (Mikkelsen*, et al.*, 2003), and a larger electron density blob was identified as the phosphates bound to this motif (Figure 5A). Most of the structure was built in this map using O (Jones *et al.*, 1991). The structure was then built and refined using native 2.4 Å resolution data.

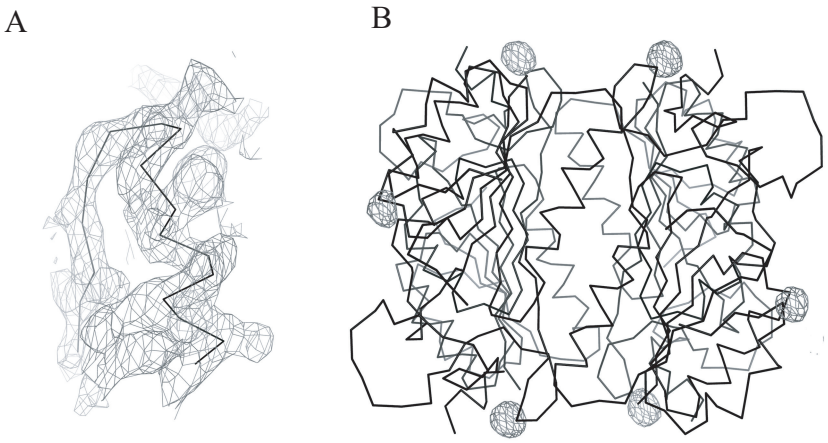

Figure 5. (A) Initial electron density map of the P-loop contoured at 1σ. (B) Tetramer with four zinc sites and two gold sites illustrated by an anomalous Fourier map contoured at 5σ. All figures containing protein structure have been made using Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA.).

2.4.4. Heavy atom sites

The heavy atom sites used to solve the structure were localized using anomalous Fourier maps with CCP4 (CCP4, 1994) . Eight stronger and six weaker sites were used in the structure determination and the eight stronger sites corresponded to eight zinc atoms bound to each subunit in the two tetramers. In addition, six weaker gold sites were found to be bound to Cys79 in six of the eight subunits (Figure 5B). The isomorphous and anomalous differences from the six gold sites and the anomalous signal from the eight zinc sites were sufficient to solve the structure. It was later shown that the human TK1 structure could be solved using zinc ion anomalous diffraction (Birringer*, et al.*, 2005).

2.5. Structure of the human TK1

The TK1 structure revealed a fold different from the earlier known structures of the dNK family. The structure is built up from an α/β and a lasso like domain containing a zinc. The α/β domain in TK1 has a central β-sheet consisting of six parallel strands surrounded by five α-helices (Figure 6A). The DALI server (Holm & Sander, 1995) revealed that the α/β domain of the structure had a high similarity to enzymes belonging to the RecA-F_1ATPase family (Sawaya*, et al.*, 1999). The active site is buried between the two domains. All structures solved in this TK1 family have been shown to be very similar. The N-terminus is flexible and starts to become ordered at Arg18, while there are only a couple of residues missing in the C-terminus for the truncated enzyme. The structure of the wild type enzyme did not reveal more of the C-terminus indicating flexibility (unpublished data). The structures of the human TK1 and *Uu*-TK were simultaneously solved, with both structures showing a high structural similarity with a root mean square deviation (RMSD) of 1.32 Å for 152 Cα-atoms.

2.5.1. Quaternary structure

The eight subunits in the crystal structure form two tetramers. The tetramer contains a central channel with mainly polar and charged residues in the interface (Figure 6D). The subunits in the tetramer have 222 symmetry. There are two types of dimer interactions in the tetramer. A helical interaction between α1 in subunit A and α1 in subunit B (Figure 6B), is strikingly similar to the dimerization of CobA (RMSD of 1.95 Å over 103 aligned residues), which has the most similar α/β domain structure to TK1 according to the DALI server (Holm & Sander, 1995). CobA catalyzes the transfer of an adenosyl moiety to corrinoid substrates (Bauer *et al.*, 2001). The second dimer interaction is connected via a range of water molecules in between two anti-parallel β-sheets in subunit A-C (Figure 6C).

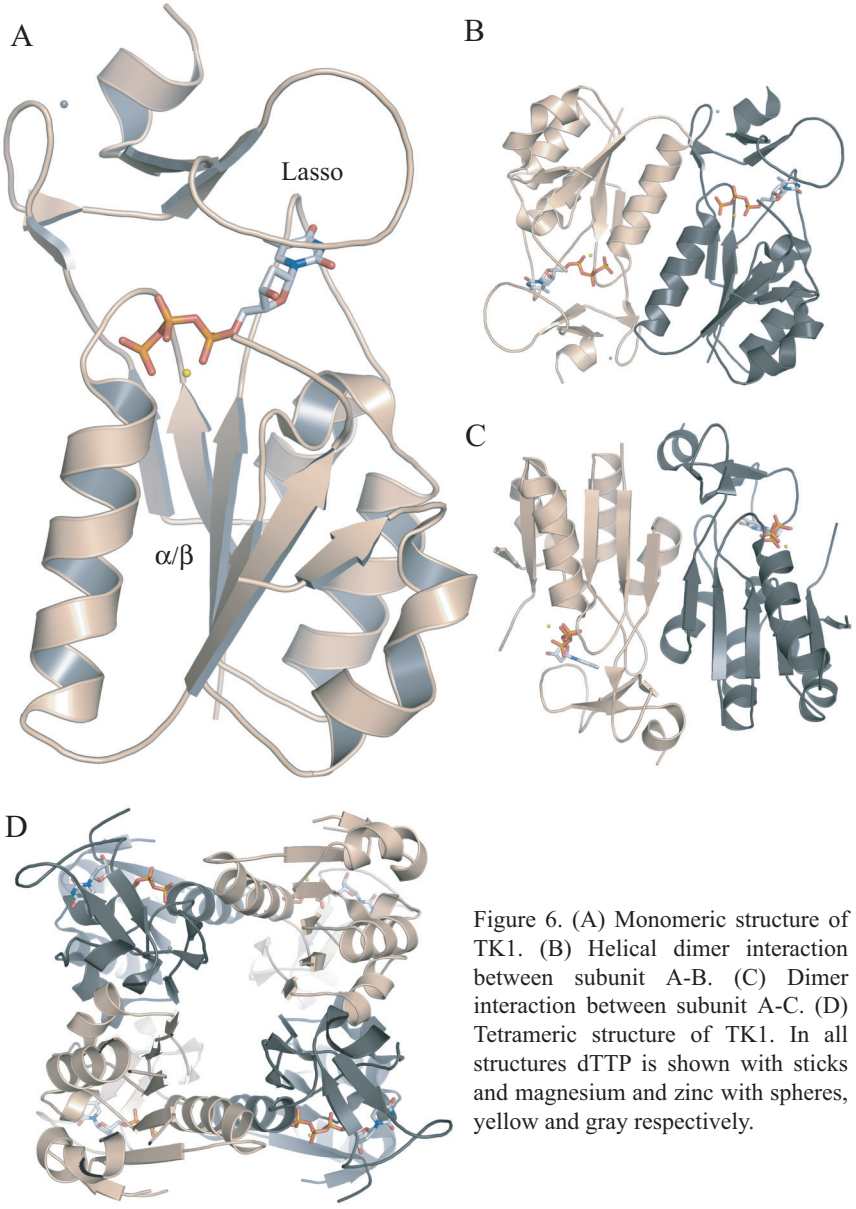

Figure 6. (A) Monomeric structure of TK1. (B) Helical dimer interaction between subunit A-B. (C) Dimer interaction between subunit A-C. (D) Tetrameric structure of TK1. In all structures dTTP is shown with sticks and magnesium and zinc with spheres, yellow and gray respectively.

2.5.2. Lasso domain

Unique for the TK1 family is a domain referred to as the “lasso domain”. This

domain has a repeated CXXC motif similar to other structural zinc motifs, where four cysteine are coordinating a zinc atom (Vallee & Auld, 1990). In some species the last cysteine in this motif is replaced by a histidine. The zinc has a structural role and is located far from the active site. The lasso domain consists of roughly forty amino acids and between the strands in the β-ribbon the lasso opens up and residues 167-179 form a long loop that covers one side of the dN site. This loop is stabilized by hydrogen bonds from main chain atoms of the tip of the loop, by a conserved Arg-Tyr couple (Figure 7), and from interactions of the dN bound to the enzyme. The crystal structure of *Ca*-TK with an empty acceptor site and an ADP bound to the donor site verify the flexibility of the loop since part of the loop is missing (Kuzin*, et al.*, 2004). The combination of an α/β domain with a zinc containing lid has been observed in bacterial adenylate kinases, where the zinc connects the tip of the lid in the adenylate kinase (Berry & Phillips, 1998; Glaser *et al.*, 1992). The role of the zinc is completely different in TK1, however, since the zinc binds at the base of the β-ribbon of the lid.

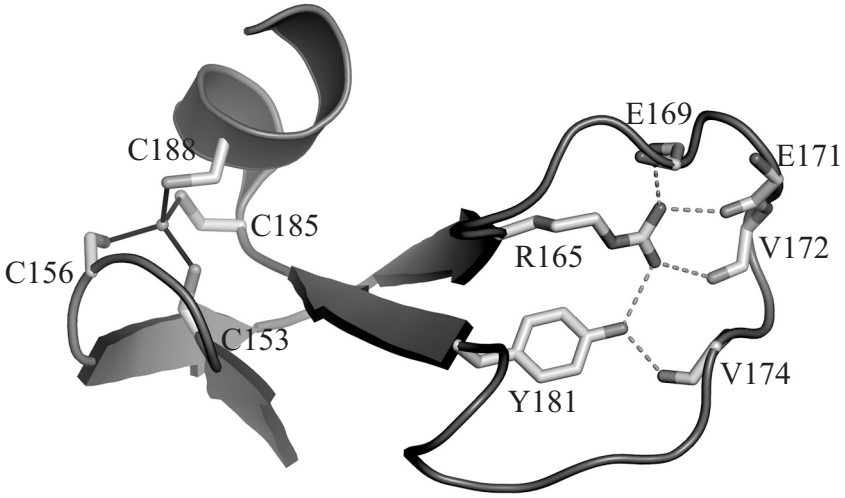

Figure 7. Lasso domain of the TK1 structure. Residues involved in binding of the zinc and stabilization of the loop are shown and numbered.

2.5.3. Acceptor site

When a substrate is bound to the enzyme the lasso loop closes down over the base of the substrate. All the hydrogen bonding interactions between the thymine base and enzyme are via main chain atoms coming from both the lasso and the α/β domain. The feedback inhibitor dTTP binds with the dT part in the acceptor site and the triphosphates to the P-loop in a manner similar to earlier observations concerning bound feedback inhibitors (Johansson*, et al.*, 2001; Mikkelsen*, et al.*, 2003). Glu98 was in proximity to the 5´oxygen of the dT moiety of the dTTP and is believed to be the catalytic base and thus may have the same mechanism as in other dNKs (Eriksson*, et al.*, 2002; Wild*, et al.*, 1997). This glutamic acid was also shown to move towards the 5´OH when only thymidine was bound in *Uu*-TK (Kosinska *et al.*, 2005). The 3´OH of the deoxyribose of dTTP is anchored via hydrogen bonds from two conserved residues, Asp58 from the α/β domain and

Gly176 from the lasso domain (Figure 8). The rather small dN binding site and the main chain interactions with the substrate base explains the narrow substrate specificity for TKs. The feedback inhibitors have been shown to bind tightly to the enzymes allowing TK1 to acquire a dTTP molecule during the purification procedure (Birringer*, et al.*, 2005; Birringer*, et al.*, 2006). This has also been observed for the human TK2 where several end products (dTTP, dCTP and dATP) were found to be bound to the purified enzyme (Barroso, Elholm & Flatmark, 2003).

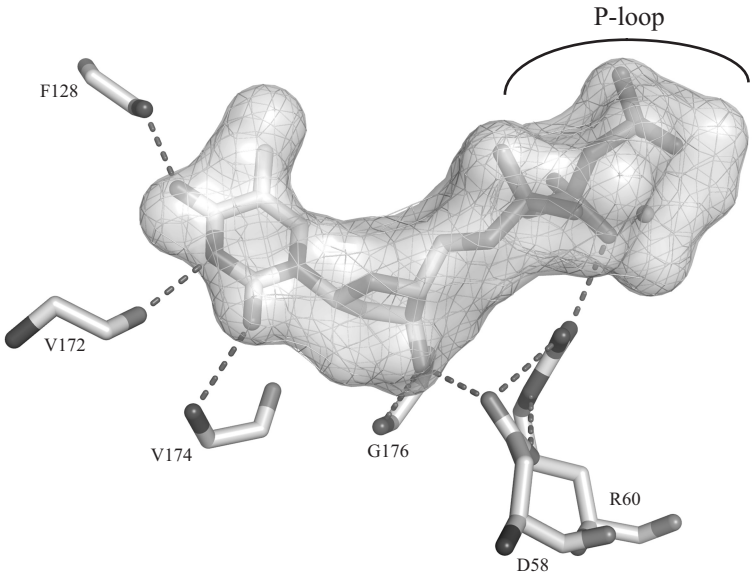

Figure 8. Phosphate acceptor site of TK1 with bound dTTP. Residues involved in binding the dN moiety are shown and numbered. The volume of the dN binding site is illustrated with a surface.

2.5.4. Comparison with other dNKs

Enzymes belonging to the TK1 family have a conserved P-loop which is the only conserved motif between the dCK/dGK and TK1 family. By superimposing the P-loop, the subsequent α1 superimpose closely on the corresponding α1 of other dNKs. The other helices do not superimpose and other dNKs have more helices, which are located differently and are longer. In particular, the substrate binding site is formed by two helix-pairs in dNKs and the lid that closes over the phosphate binding site of the phosphate donor is a helical structure in dNKs.

2.5. TK1 and drug design

In this study the structures of two TKs of human and mycoplasmic origin were solved. The nearly identical active sites in the enzymes complicate the possible design of anti-mycoplasmic agents targeting only the *Uu*-TK enzyme. In the active site there is only a Thr to Ser substitution in the *Uu*-TK. The human TK1 has been shown to accept modifications at the 3´OH position such as AZT. In addition, boronated compounds with N3 substitutions of the thymine base are phosphorylated by TK1. The N3 position points towards the interface between the α/β and the lasso domain and a partly closed lasso domain could be a reason for accepting these substrates.

3. *Drosophila melanogaster* deoxyribonucleoside kinase, *Dm*-dNK (Paper II)

3.1. Aim of study

In a random mutagenesis investigation of *Dm*-dNK a double mutant, N45D/N64D, revealed increased sensitivity toward several NAs when the gene was transformed into a TK deficient *E. coli* strain. Furthermore, decreased inhibition with the end product dTTP was observed (Knecht, Munch-Petersen & Piskur, 2000). Interestingly, both these mutations were located far from the active site and this finding initiated further investigations. Structural and biochemical characterizations were carried out for the separate mutations (N45D and N64D) to reveal the basis for the obtained behavior of the double mutant.

3.2. Background

Dm-dNK differs from other dNKs since it is capable of phosphorylating all natural substrates for DNA synthesis, but with higher substrate specificity towards pyrimidines. *Dm*-dNK has shown higher catalytic rates both with natural substrates and nucleoside analogs compared to human dNKs and HSV1-TK (Johansson*, et al.*, 1999; Munch-Petersen, Piskur & Søndergaard, 1998). Truncating the last twenty residues of *Dm*-dNK revealed similar K_m values but a higher turn over number than the wild type enzyme (Munch-Petersen*, et al.*, 2000). For both the crystal structure and gel filtration experiments of *Dm*-dNK, a homodimeric protein has been observed (Johansson*, et al.*, 2001; Knecht*, et al.*, 2002a). Although *Dm*-dNK can phosphorylate dT, dC, dG and dA, its only potent feedback inhibitor is dTTP (Knecht, Munch-Petersen & Piskur, 2000; Knecht*, et al.*, 2002a).

3.3. Mutational studies

To improve phosphorylation of nucleoside analogs, *Dm*-dNK has been thoroughly investigated and several mutational studies have been carried out. Knecht, *et al.* (2002b) identified three active site residues using the crystal structure of dGK to be the basis for a more purine specific enzyme. The corresponding residues (V84A, M88R and A110D) in *Dm*-dNK were mutated and the triple mutant turned out to be more purine specific than the wild type *Dm*-dNK (Knecht *et al.*, 2002b). Recently it was demonstrated that the M88R mutant also showed increased sensitivity towards the purine analog, 9-β-D-arabinofuranosyl guanine (araG) (Solaroli *et al.*, 2006a). Several mutants increasing the sensitivity toward nucleoside analogs were identified using high-frequency random mutagenesis. Especially one double mutant, N45D/N64D, displayed a decreased lethal dose $(LD)_{100}$ towards several nucleoside analogs when the gene was transformed into a TK deficient *E. coli* strain (Knecht, Munch-Petersen & Piskur, 2000).

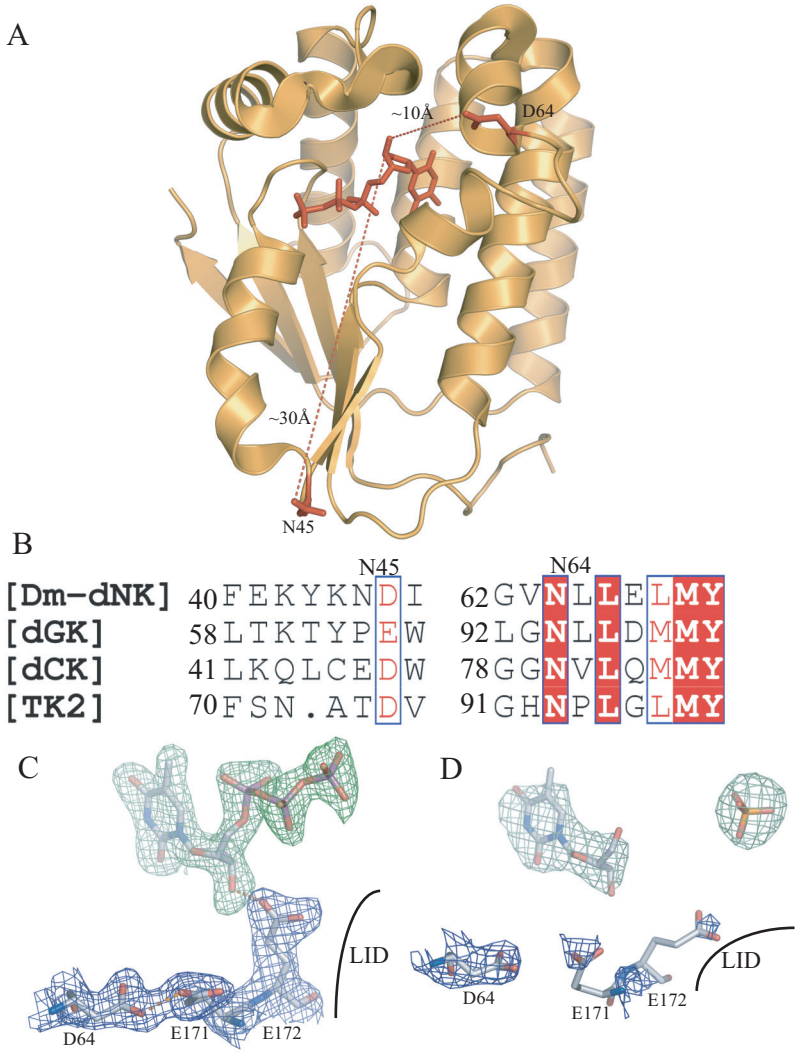

Figure 9. (A) Structure of *Dm*-dNK N64D with bound dTTP and positions N45 and D64 in sticks and colored red. (B) Structure-based sequence alignment for the regions surrounding the mutations for *Dm*-dNK (accession number: Q9X7T6), dGK (accession number: Q16854), dCK (accession number: P27707) and TK2 (accession number: O00142). (C) and (D) 2Fo-Fc electron density map contoured at 1σ for residues D64, E171, and E172 in blue. Fo-Fc electron density map contoured at 3σ (before the ligands were included in the refinement) for dTTP and dT and a sulfate ion in green.

3.3.1. Effects of N45D/N64D

An increase in sensitivity towards AZT and 2′,3′-dideoxycytidine (ddC) of 316-fold and 11-fold, respectively, in LD_{100} values for the N45D/N64D mutant were

shown in a TK deficient strain (Knecht, Munch-Petersen & Piskur, 2000). Although both mutations were located far from the active site ~30Å for N45D and ~10Å for the N64D (Figure 9A), an impact on the sensitivity towards nucleoside analogs was observed. Another characteristic observed with the double mutant was decreased feedback inhibition by dTTP and lower activity concerning natural substrates (Knecht, Munch-Petersen & Piskur, 2000).

3.4. N64D mutant

3.4.1. Effects of the N64D mutant

Asn45 is a non-conserved residue while Asn64 is conserved among dNKs (Figure 9B). To reveal the significance of the two point mutations they were investigated separately. The mutation responsible for the changed behavior of the *Dm*-dNK turned out to be the N64D, which alone showed similar patterns as the double mutant. The N64D mutant required the highest concentration of dT for growth in a TK deficient *E. coli* strain followed by N45D/N64D and N45D. The N64D mutant showed a 300-fold decrease in LD_{100} towards AZT, similar to the double mutant. The same pattern was observed concerning the feedback inhibition with dTTP. N45D behaved as the *Dm*-dNK while in the N64D mutant the inhibition was almost abolished. To investigate the observed changes, the *Dm*-dNK N64D mutant was co-crystallized together with the substrate, dT, and the feedback inhibitor, dTTP.

3.4.2. Structural basis for the changed behavior of the N64D mutant

The crystal structure of the N64D-dTTP complex did not display any differences compared to the wild type structure. Surprisingly there was still a hydrogen bond interaction between Asp64 and Glu171 despite the negative charges (Figure 9C). The LID is kept in place by several interactions with the phosphates of the dTTP molecule. The unfavorable interaction between an aspartic acid and a glutamic acid has been observed in several cases (Flocco & Mowbray, 1995). The decreased feedback inhibition by dTTP can probably be explained by the energetic cost holding these two carboxylates together. In the crystal structure of N64D-dT complex the situation is different. When the LID is not held in place with the strong interactions with the phosphates the two carboxylates repel and destabilize this part of the structure (Figure 9D). The unordered LID structure leads to the loss of a hydrogen bond between Glu172 and the 3´OH group of the deoxyribose. The loss of this bond creates a more open dN acceptor site and more room for the bulkier AZT molecule, which are displayed by the increased sensitivity towards AZT. This would also explain the increased $K_{0.5}$ with the natural substrates (dT, dC, dA and dG).

3.5. Medical/industrial use

Modifying *Dm*-dNK to obtain an enzyme more specific towards nucleoside analogs and less sensitive for feedback inhibition could be a potential candidate as a suicide gene in combined gene/chemotherapy, in the same fashion as HSV1-TK. Another use of *Dm*-dNK could be for large-scale production of dNTPs or phosphorylated analogs (Munch-Petersen*, et al.*, 2000).

4. Mycoplasma mycoides deoxyadenosine kinase, *Mm*-dAK (Paper III)

4.1. Aim of study

A significant amount of structural information concerning dNKs has been obtained in the last decade, but no structure of a bacterial enzyme belonging to the dCK/dGK family has been solved. *Mm*-dAK was characterized in an earlier study and crystallographic studies were then initiated to explore differences between the bacterial enzyme and its human counterparts. Additionally, activity measurements of several NAs were performed to identify potential leads for future drug design.

4.2. Background

Bacterial dNKs vary in number, and in mycoplasmas there are two different enzymes, a TK and a dAK. Among mycoplasmic TKs, *Uu*-TK has been characterized and its structure has been determined (Carnrot*, et al.*, 2003; Kosinska*, et al.*, 2005; Welin*, et al.*, 2004). The most studied enzyme belonging to the dCK/dGK family in mycoplasmas is *Mm*-dAK. This was the first bacterial dNK belonging to this family to have its crystal structure determined.

Mycoplasmas are the smallest self replicating organisms and are further characterized by a lack of a cell wall and a low G+C content. This group of organisms can cause disease in animals, plants, and insects (Razin, Yogev & Naot, 1998). They can infect a broad range of hosts, where *Mm* infects cattle and buffalo and can cause a respiratory disease called contagious bovine pleuropneumonia(Pilo, Frey & Vilei, 2006). Human pathogens such as *Uu* and *Mycoplasma pneumoniae* (*Mp*) can cause urinary tract infections and pneumonia (Hammerschlag, 2001; Pollack, 2001). Mycoplasmas are unable to produce purine and pyrimidine bases de novo and are thus dependent on the salvage enzymes for production of NTP/dNTPs (Pollack, Williams & McElhaney, 1997). In *Uu* no genes for RNR were found and no genes for nucleotide diphosphate kinase have been discovered in mycoplasmas in general (Glass *et al.*, 2000). The *Mm*-dAK is capable of phosphorylating, in order of catalytic efficiency, dA, dG, and dC. In addition several important nucleoside analogs can be phosphorylated while ribonucleosides are barely phosphorylated at all.

4.3. Structure of *Mm*-dAK

The sequences of *Mm*-dAK and dCK are ~25% identical, indicating that they have similar structures. Initial attempts to solve the structure of *Mm*-dAK with molecular replacement failed, so purification of selenomethionine (SeMet) substituted protein was initiated. Several SAD data sets of SeMet-crystals were collected without any success. At the time these experiments were taking place new molecular replacement trials with a new version of Phaser (McCoy *et al.*, 2005) were carried out and a correct solution was found. The *Mm*-dAK structure was solved and the structure revealed high structural similarity with dCK, dGK and *Dm*-dNK. The structure of *Mm*-dAK revealed a five stranded parallel β-sheet surrounded by eight α-helices (Figure 10A). *Mm*-dAK is a dimer like other enzymes belonging to the dCK/dGK family.

4.3.1. Mm-dAK complexes

Three different complexes of *Mm*-dAK were determined, with the feedback inhibitors dATP and dCTP, and the products dCMP + dCDP. The overall structure between the complexes is relatively unchanged except in a few regions. A tyrosine moves ~ 8Å in the pyrimidine complexes compared to the dATP complex to make hydrophobic interactions with an α-helix of the C-terminus from a symmetry related molecule. The *Mm*-dAK-dATP complex was crystallized in space group C2 with six molecules in the asymmetric unit, while the pyrimidine complexes were crystallized in $P3_1$ with a dimer in the asymmetric unit. In all complexes hexamers of three dimers can be generated. The arrangements of the dimers, however, are different and are believed to be crystal artifacts. This also coincides with *Mm*-dAK eluted as a dimer during gel filtration chromatography. This is in agreement with other known dimeric structures of the dCK/dGK family.

4.3.2. Mm-dAK-dATP

The dATP molecule was known from kinetic data to be a potent inhibitor of *Mm*-dAK and was therefore used in crystallization trials. The dATP molecule binds in a reverse mode with its dN moiety in the acceptor site and the phosphates occupying the P-loop. The adenine base makes stacking interactions with Met57 and Phe86, where the methionine corresponds to a phenylalanine in dGK and dCK. The conserved Gln54 that interacts with the tip of the base is in most subunits hydrogen bonded to N6 position of the adenine (Figure 10B). Tyr42 is stacked against the C2 position of the base. The Mg^{2+} observed was bound in proximity to Glu150 which corresponds to the opposite side compared to earlier observations in TK1 and *Dm*-dNK (Mikkelsen*, et al.*, 2003; Welin*, et al.*, 2004).

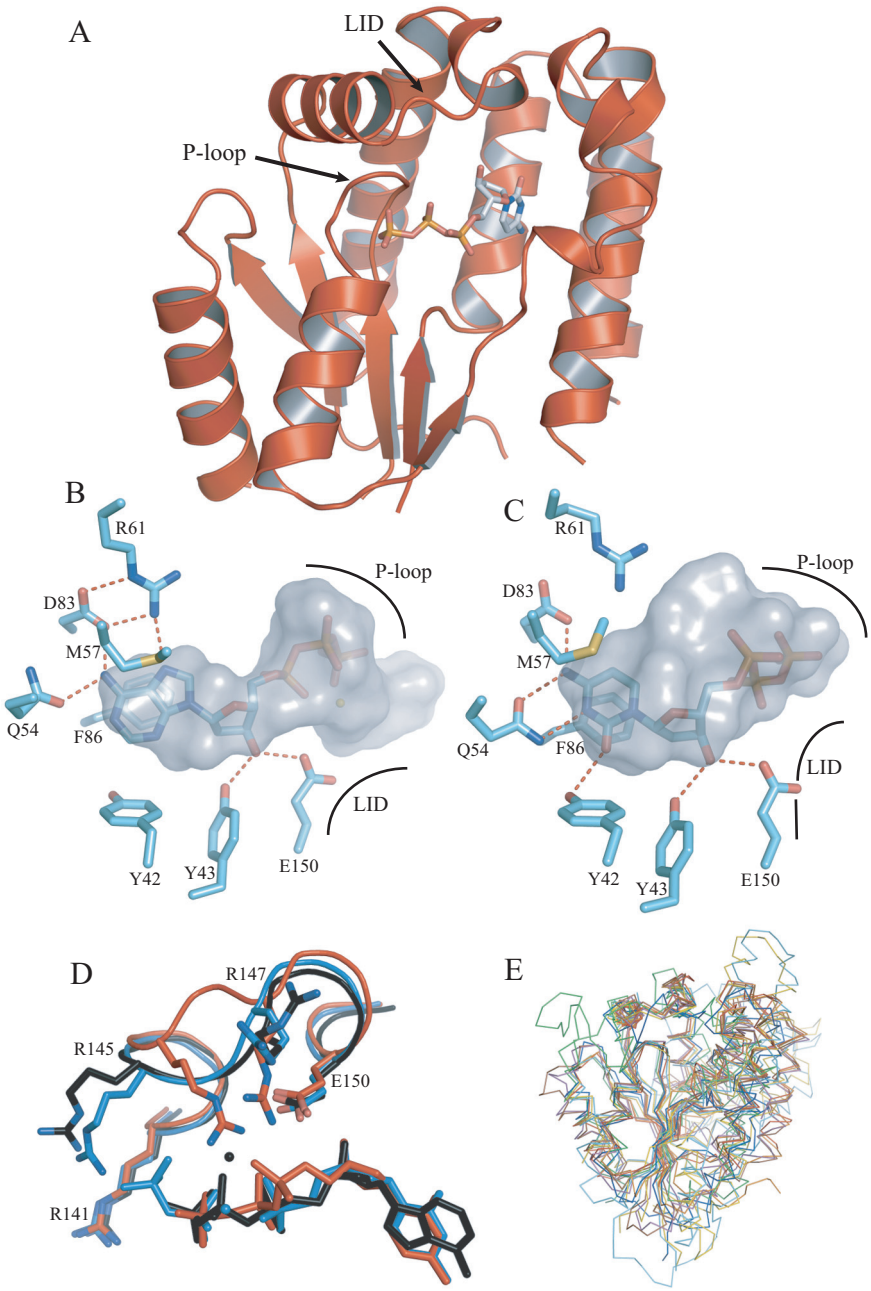

Figure 10. (A), Monomer of the dimeric *Mm*-dAK structure in complex with dCTP, illustrating the location of the conserved P-loop and LID motif. Comparison of feedback inhibitor complexes (B) dATP and (C) dCTP, volume of the dN binding sites are illustrated by surfaces. (D) Comparison of the LID region, *Mm*-dAK-dCTP, *Mm*-dAK-dATP and *Mm*-dAK-dCMP-dCDP are shown in red, black and blue respectively. Magnesium ions are shown in spheres and have the same color as the complex (E) Superimposition of several other similar protein structures color coded in Table 2.

4.3.3. Mm-dAK-dCTP

Kinetic studies using dCTP revealed that this nucleotide works as a phosphate donor. So the binding of dCTP as a feedback inhibitor was not expected. The dCTP binds in a similar fashion as the dATP molecule, though there are some small side chain alterations in the acceptor site. The conserved glutamic acid has moved closer into the dN substrate binding pocket and interacts with the N3 and N4 position of the cytosine. Tyr42 is now hydrogen bonded to the 2-oxo of the cytosine (Figure 10C). No Mg^{2+} was observed in this complex.

4.3.4. Mm-dAK-dCMP-dCDP

Since dCTP could work as a phosphate donor, crystallization trials using both dC and dCTP were performed. The electron density from this complex was interpreted as two products, dCMP and dCDP bound to the active site. The deoxycytidine part of dCMP binds like the dN moiety of dCTP, while the dCDP binds to the P-loop. The deoxycytidine part of the dCDP is very flexible and only the diphosphate is visible in the density. Weak density for a Mg^{2+} was seen in this complex as well, but bound to the opposite side compared to the *Mm*-dAK-dATP complex. In this complex the Mg^{2+} is bound in proximity to Glu77.

4.3.5. LID region

The LID region with the conserved motif [RXXXRXRXXE] is present in all dCK/dGK family enzymes. When the P-loop is occupied by a triphosphate the conserved arginines are usually positioned towards the negatively charged phosphates. This binding mode has been observed in *Dm*-dNK in complex with dTTP and in dGK in complex with dATP[1] (Johansson*, et al.*, 2001; Mikkelsen*, et al.*, 2003). In *Mm*-dAK the LID region takes different conformations in the different complexes. The Arg141 in the LID motif is positioned in the same conformation in all three complexes as well as Glu150. This glutamic acid is interacting with the 3´OH of the deoxyribose moiety. Residues 145-147 are more flexible but the rest of the LID is positioned in the same orientation. For the *Mm*-dAK-dCTP complex the main chain of the LID is located 4-5 Å further away from the phosphates compared with other *Mm*-dAK complexes (Figure 10D). A possible explanation for this is the unusual position for the bound magnesium, which might push the arginines away from the phosphates.

[1] Originally the structure of dGK was described in complex with ATP. After later evidence the ligand has been reinterpreted as a dATP.

Organism	PDB-code	RMSD (Å)	Aligned residues	Color
M.mycoides	2JAQ	-	-	Red
D.melanogaster	1OE0	1,46	156	Orange
H.sapiens	1P61	1,75	172	Magenta
H.sapiens	2OCP	1,75	175	Brown
Varicella Zooster Virus	1OSN	2,05	147	Cyan
S.cerevisiae	3TMK	2,2	151	Blue
HSV1	1P7C	2,2	169	Yellow
B.stearothermophilus	1ZIN	2,4	137	Green

Table 2. Structural comparison of *Mm*-dAK with similar structures, superimposition is shown in Figure 10E. TMPK – thymidylate kinase, AMPK – adenylate kinase.

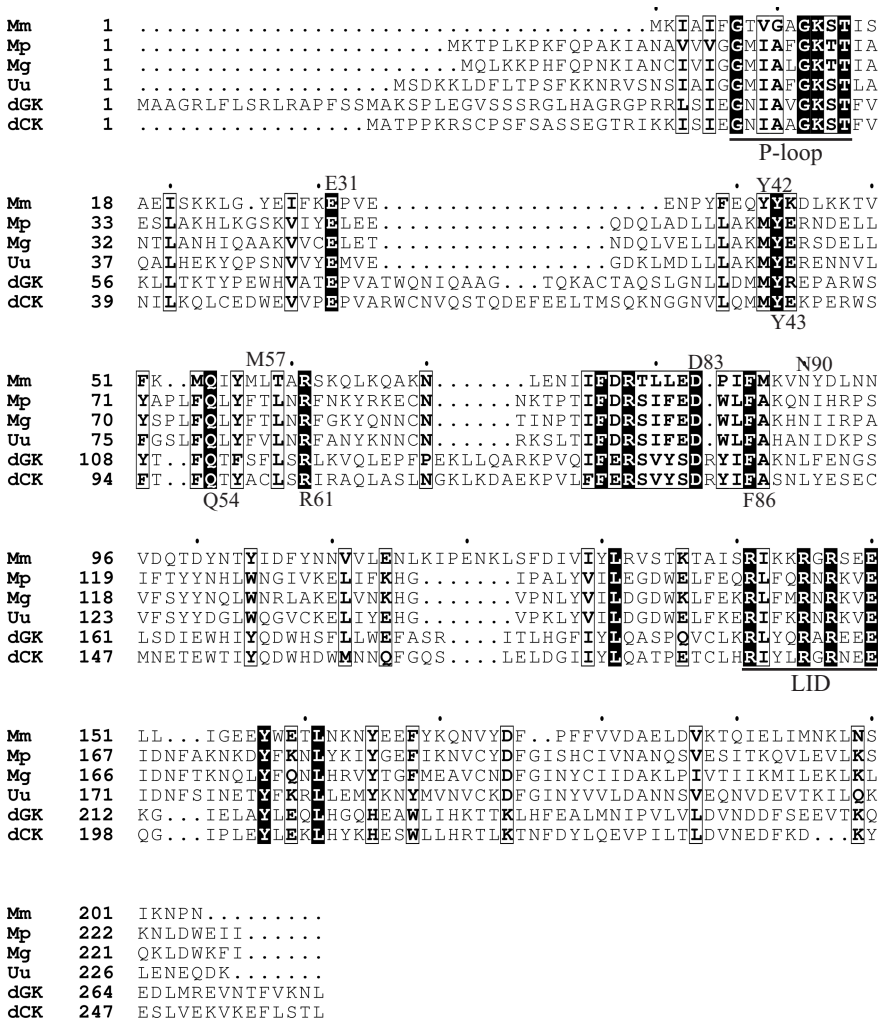

Figure 11 (previous page). Amino acid sequence alignment of *M. mycoides* (Mm, accession no: Q93IG4), *M. pneumoniae* M129 (Mp, accession no: P75396), *M. genitalium* G37 (Mg, accession no: P47510), *U. urealyticum* (Uu, accession no: Q9PR59), human deoxyguanosine kinase (dGK, accession no: Q16854) and deoxycytidine kinase (dCK, accession no: P27707). The P-loop and LID region and amino acids in proximity of the acceptor site with numbering from *Mm*-dAK are marked. Residues that differ and residues that are conserved with *Mm*-dAK numbering are shown above and below the alignment respectively. Black and white boxes refer to identical and similar residues respectively. The sequence alignment was made using ClustalW (Chenna *et al.*, 2003) and ESPript (Gouet *et al.*, 1999).

4.3.6. Comparison with other dNKs and (d)NMPKs

The high sequence similarity between dCK, dGK and *Dm*-dNK indicated that the structures of these enzymes would be similar. Superimposition of these enzymes to the *Mm*-dAK structure showed an RMSD of ~1.5-1.8 Å. Several (d)NMPKs and viral TKs were identified as similar structures using the DALI server (Holm & Sander, 1995). All structures displayed in table 2 were superimposed and clearly illustrates the similarity of the core structures (Figure 10E).

4.3.7. Mycoplasmic enzymes belonging to the dCK/dGK family

When sequences from human pathogenic mycoplasmas are compared they reveal a sequence identity of ~22-25%. Most of the residues in the active site proximity are conserved compared to its human counterparts with three exceptions: (i), Tyr42; (ii) Met57; and (iii) Asn90 (Figure 11). (i), a tyrosine in this position is not observed in other mycoplasma species or in the human counterparts. (ii), Met57 varies among the different sequences: it is a phenylalanine in the other mycoplasmas, a serine in dGK and an alanine in dCK. The residue in this position is of key importance for substrate specificity of the enzyme. Enzymes having a hydrophobic amino acid in this position, like *Mm*-dAK and dCK, show broader substrate specificity. Both of these enzymes have higher activity (k_{cat}/K_m) towards dA, dG and dC, although dGK can phosphorylate dC to some extent (Eriksson*, et al.*, 2002). The cause for the specificity is a conserved aspartic acid that interacts with the N6 position of adenine and N4 of cytosine. In dGK a serine is hydrogen bonded to the aspartic acid, hence this amino acid is not free to adopt different conformations depending on the substrate. In *Mm*-dAK and dCK this aspartic acid is not locked in one position and can adopt different conformations depending on the substrate. (iii), Asn90 is conserved among the mycoplasmic sequences shown, while it is a leucine in dGK and dCK. The asparagine in *Mm*-dAK interacts with the Tyr42 and is situated 5.5 Å from the edge of the base.

4.4. Mm-dAK and potential drug design

As bacteria increase their antibiotic resistance we need to develop new anti-bacterial agents (Rice, 2006). Several pathogenic gram positive bacteria possess a dNK belonging to the dCK/dGK family, which could be potential targets for anti-bacterial agents. In this study we investigated a mycoplasmic dNK, a dAK from *Mm*. In *Uu*, no genes corresponding to RNR have been found which render it

dependent on the salvage enzymes and thus a good target for drug design (Glass, *et al.*, 2000). With the structure of *Mm*-dAK, we have a model structure for future design of new anti-mycoplasmic agents. By structural comparison of *Mm*-dAKs with its human counterparts dCK and dGK, we have tried to reveal differences that can be exploited in the design of anti-bacterial agents. Modifications at the C2 position of an adenine substrate would be of interest for agents against pathogenic mycoplasmas since they possess a Met instead of a Tyr and thus have more space around this position. A C2 modification could then interact with the conserved Asn in myoplasmas.

5. Human phosphoribosyltransferase domain containing 1, PRTFDC1 (Paper IV)

5.1. Aim of study

This study was carried out at the Structural Genomics Consortium (SGC) at Karolinska Insitutet, Stockholm. PRTFDC1 is a human enzyme of unknown function highly similar to the human hypoxanthine guanine phosphoribosyltransferase (HPRT). By structural determination and biochemical studies our aim was to elucidate the potential function of PRTFDC1.

5.2. Background

Recently, a homolog of HPRT, PRTFDC1, was investigated by bioinformatical approaches (Keebaugh, Sullivan & Thomas, 2006; Keebaugh, Sullivan & Thomas, 2007; Nicklas, 2006). This gene has been classified as having an unknown function. As the name indicates it has a domain similar to phosphoribosyltransferases. PRTFDC1 are sequentially very similar to HPRT with a sequence identity of ~65%. Different HPRTs, both mammalian and bacterial, have high sequence similarities. Comparing human HPRT with a bacterial (*E. coli)* HPRT shows a sequence identity of ~29% and a similarity of ~44%. Among different HPRTs there are eleven invariant residues in proximity to the active site, with an exception for *Giardia lamblia* where only nine of these residues are conserved (Craig & Eakin, 2000). None of the PRTFDC1 sequences investigated by Keebaugh, *et al.* (2007) possessed all the invariant residues (Keebaugh, Sullivan & Thomas, 2007). In the human HPRT an aspartic acid at position 137 is believed to act as the catalytic acid/base and mutational studies of this residue to an asparagine showed a 30-fold reduction in catalytic efficiency (Canyuk, Focia & Eakin, 2001; Xu & Grubmeyer, 1998). In *Leishmania donovani* adenine phosphoribosyltransferase (APRT) the corresponding residue is an alanine, indicating a different mechanism or a movement of a distant amino acid during catalysis (Craig & Eakin, 2000). Lys165 (HPRT numbering) is critical for discriminating substrates, since the NZ of the lysine is forming a hydrogen bond with the 6-oxo of the base. If this lysine is mutated to serine additional activity with adenine is obtained (Munagala, Chin & Wang, 1998).

5.3. Structure of PRTFDC1

Crystals of PRTFDC1 belonged to the space group P321 and had two subunits in the asymmetric unit. PRTFDC1 revealed a structure similar to other known HPRTs. Despite co-crystallization with PRPP, electron density corresponding to a GMP was found in the active site. This indicates that the GMP was obtained during expression. A similar result was observed for xanthine phosphoribosyltransferase from *Bacillus subtilis* (Arent *et al.*, 2006). The structure of PRTFDC1 can be divided into two domains, a core and a hood domain. (Eads *et al.*, 1994). The core domain consists of a six stranded twisted parallel β-sheet with the β-strands in the order 187634. Three α-helices surround the central β-sheet. β4

in the β-sheet is extended into a β-ribbon with β5 and this part of the structure is referred to as loop II. The hood domain is mainly built up by parts from the N- and C-terminus (Figure 12A). Using symmetry operations the two monomers in the asymmetric unit can generate a tetramer (Figure 13A).

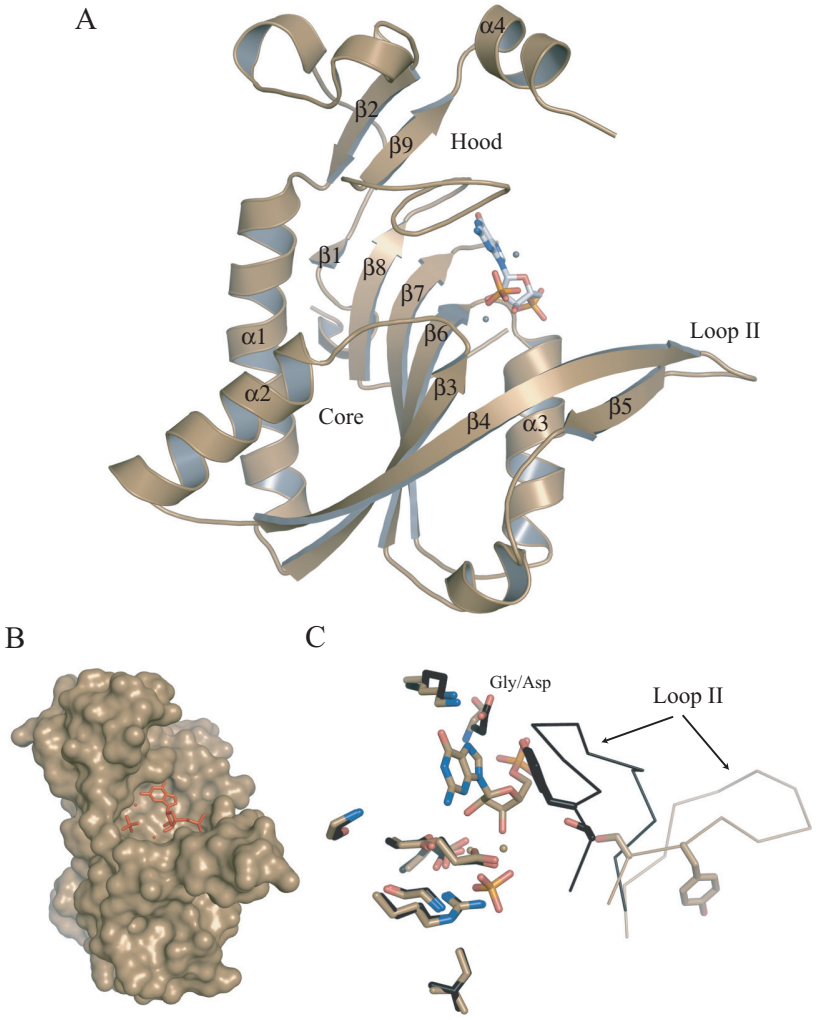

Figure 12. (A) Monomer of PRTFDC1 with GMP, a phosphate ion and two calcium ions bound. (B) Surface representation of PRTFDC1, GMP, calcium ions and the phosphate ion are colored in red. (C) Active site comparison of PRTFDC1 and HPRT (PDB-code: 1BZY) in brown and black respectively. GMP, a phosphate ion and two calcium ions from the PRTFDC1 structure are shown in brown. Loop II is shown with Cα-trace in both structures. Residues are shown with sticks and the Gly/Asp difference between HPRT and PRTFDC1 are shown.

5.3.1. Loop II

This loop encloses the active site in several transition state analog structures, but is otherwise flexible and not observed (Focia, Craig & Eakin, 1998; Shi *et al.*, 1999). Deletion of seven residues of loop II in *Trypanosoma cruzi* HPRT showed practically unchanged K_m values for the substrates Hx and G while the catalytic efficiency decreased 240- to 1400-fold compared to the wild type. These results indicate that loop II is not directly involved in binding substrates but rather in catalysis (Lee *et al.*, 2001). In PRTFDC1 this loop is clearly visible in one subunit, where the loop is formed as a long anti-parallel β-ribbon. The loop, however, does not cover the active site but stretches out from the protein forming interactions with a symmetry related molecule (Figure 13B). Two mutations in this region, S103R and S109L, lead to gout (Sculley *et al.*, 1992).

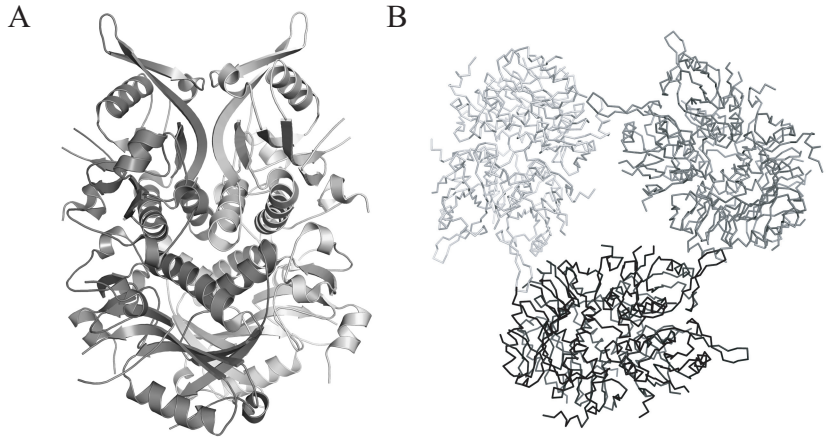

Figure 13. (A) tetramer generated from symmetry related molecules. (B), interactions of loop II with symmetry related molecules.

5.3.2. Invariant residues

Among eukaryotic HPRTs, eleven residues flanking the active site are invariant, while none of the compared PRTFDC1 sequences showed complete conservation. The most common substitution for PRTFDC1s were the Asp137 to Gly (Keebaugh, Sullivan & Thomas, 2007). A comparison of the human HPRT and PRTFDC1 shows nearly identical positions of these residues, with the exception of the Ser-Tyr couple which swings in to cover the active site in HPRT while it is pointing away in the PRTFDC1 leaving the active site very open (Figure 12B and C). By superposing the PRTFCD1 with other known HPRTs illustrates that the core structures are well conserved and the major differences between the structures are the flexible loop II.

5.3.3. Is PRTFDC1 an active enzyme?

Since PRTFDC1 has a Gly substitution at the position for the believed catalytic residue it was proposed to have lost its phosphoribosyltransferase activity. The mouse PRTFDC1 was recently discovered to be inactive and hypothesized to be a cause for the development of the neurological symptoms of Lesch-Nyhan disease since HPRT deficient mice did not display these symptoms (Keebaugh, Sullivan & Thomas, 2007). To investigate the potential function of PRTFDC1, a basic spectrophotometric assay was utilized (Hill, 1970). The forward reaction showed activity using Hx and G together with PRPP with Hx as the preferred substrate. Hx as a favorable substrate also coincides with the performed thermofluor assay (Ericsson *et al.*, 2006), where the highest increase in melting temperature was obtained using IMP. In the reverse reaction no detectable activity with IMP as a substrate was observed. The low or not detectable activities might be an effect of the pre bound GMP and insensitivity of the spectrophotometric assay.

6. Conclusions and future perspectives

In this thesis structural studies of salvage enzymes in the nucleotide metabolism were investigated. Three new structures, human TK1, a mycoplasmic dAK, and the human HPRT homolog, PRTFDC1, were solved and investigated. In addition a structural study of a *Dm*-dNK mutant displaying changed behavior was carried out.

6.1. Human TK1

The human TK1 and a mycoplasmic TK were the first enzymes belonging to the TK1 family to be solved, and revealed a structure different from earlier known dNKs. Comparison of the human and bacterial TK showed high structural similarity between the enzymes. The TK structure is composed of two domains, an α/β domain similar to RecA-F_1ATPase family and a second domain referred to as the lasso domain. The lasso domain closes down over the active site and is stabilized by a structural zinc coordinated by four cysteines. The structure provides an explanation for the narrow substrate specificity of TKs and will be a good starting point for the design of new NAs or potential inhibitors for the mycoplasmic enzyme. To study interactions between TK1 and NAs, further structural investigations are needed. How are NAs with modifications such as, 3´OH of the deoxyribose and the N3 position of the thymine moieties bound to the protein?

6.2. *Dm*-dNK N64D

A double mutant N45D/N64D in *Dm*-dNK was found using a directed evolution study, and displayed an increased sensitivity towards AZT when transformed into a TK deficient *E. coli* strain. Furthermore the double mutant displayed a decreased inhibitory effect with the end product dTTP. Kinetic studies revealed that the N64D mutant was responsible for the changed behavior. Structural studies with a bound substrate revealed a destabilized LID region. The hydrogen bond between Asp64 and Glu171 was lost, leading to a flexible LID region, and a weaker binding of dT. The flexible LID region makes the substrate binding site slightly bigger which may give more space for the AZT molecule to bind. In the complex with the end product dTTP, Asp64 and Glu171 is still hydrogen bonded. This observation revealed that the decreased inhibition can be explained by the high energy cost of keeping these two carboxylates together. Modifying *Dm*-dNK into an enzyme displaying increased activity towards analogs while the activities of natural substrates are decreased is of interest in suicide gene/chemotherapy using the *Dm*-dNK gene. Can more mutations increase sensitivity and decrease activity towards natural substrates even further?? How is the *Dm*-dNK mutant behaving in vivo??

6.3. *Mm*-dAK

Mm-dAK represents the first bacterial dNK belonging to the dCK/dGK family. The *Mm*-dAK is structurally similar to its human counterparts dCK and dGK.

Three complexes of the protein were obtained with two feedback inhibitors, dATP and dCTP, and two products, dCMP and dCDP. *Mm*-dAK prefers the substrates dA, dG, and dC, in order of catalytic efficiency. The crystal structure co-crystallized with dCTP, which was supposed to work as a phosphate donor, was surprisingly bound as a feedback inhibitor. The *Mm*-dAK structures, compared with their human counterparts, revealed residues important for substrate specificity. Here we have combined structural and biochemical data to find potential leads for future development of new anti-bacterial agents towards different pathogenic mycoplasma. How does NAs bind to the protein?? How is dG bound to dNKs?? These are questions that are still unanswered and hopefully future studies will reveal these answers.

6.4. PRTFDC1

The human homolog of HPRT, PRTFDC1 was classified as having an unknown function, although it possessed a phosphoribosyltransferase domain. To elucidate the potential function of PRTFDC1 both structural and biochemical data were obtained. The structure revealed a bound GMP in the active site despite co-crystallization using PRPP. A spectrophotometric assay displayed some activity using the substrate Hx and G in the forward assay while no activity was observed for the reverse reaction IMP. A thorough kinetic investigation, using more sensitive assays is needed to reveal the substrate specificity and the activity of PRTFDC1, for a precise comparison of HPRT. There are still many unanswered questions concerning the biological role of PRTFDC1. Among those that need to be answered are: Is the activity of PRTFDC1 enough for compensation of an inactive or partially active HPRT? Does PRTFDC1 have any connection to the Lesch-Nyhan disease?

These questions are beyond the scope of this thesis though the experiments we describe here are a necessary foundation to finding solutions to these important issues.

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