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Review

Structure and function of cellular deoxyribonucleoside kinases

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Abstract. Deoxyribonucleoside kinases phosphorylate deoxyribonucleosides, a crucial reaction in biosynthesis of DNA precursors through the salvage pathway. Their medical interest stems from their activation of a number of anticancer and antiviral drugs such as 2-chloro-2′-deoxyadenosine, azidothymidine and acyclovir. Here we review what is presently known about each of the mammalian kinases as well as some other members of the deoxyribonucleoside kinase family. A description of the biochemical properties of the enzymes is followed by an overview of the structural studies made on this family of enzymes, including the catalytic mechanism as well as the mechanism for feedback inhibition. A presentation of homology models of other proteins in the family is made and, finally, the determinants of substrate and substrate analog specificities are described.

Key words. Deoxyribonuncleoside kinases; substrate specificity; phosphoryl transfer; nucleoside analogs; feedback inhibition; biochemical properties.

Introduction

DNA precursors are made from ribonucleotides, the precursors of RNA synthesis [1] or from deoxyribonucleosides that originate from food or degraded cells [2]. The deoxyribonucleosides are transferred across the cell membrane by transporter proteins [3] and are then phosphorylated through the salvage pathway. The initial step is carried out by deoxyribonucleoside kinases, which catalyze the phosphoryl transfer from ATP (or other ribonucleoside triphosphates) to form deoxyribonucleoside monophosphates ([2], and references therein). The charged monophosphates become trapped inside the cells and can be further phosphorylated to DNA precursor triphosphates. The deoxyribonucleoside kinases are usually the rate-determining enzymes in the salvage pathway. There are four deoxyribonucleoside-specific kinases in mammalian cells and tissues [2], the cytoplasmic enzymes thymidine kinase (TK1) and deoxycytidine kinase (dCK), and the mitochondrial enzymes thymidine kinase (TK2) and deoxyguanosine kinase (dGK). These four kinases have distinct but overlapping specificities. In contrast, the fruitfly *Drosophila melanogaster* was recently

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found to have a multisubstrate deoxyribonucleoside kinase (dNK) with the ability to phosphorylate all natural deoxyribonucleosides with high efficiency [4, 5]. dNK is apparently the only deoxyribonucleoside kinase present in *Drosophila* [4]. A summary of the specificities and the expression pattern of these enzymes will be presented in this review.

Based on their amino acid sequences, the known deoxyribonucleoside kinases can be roughly divided into three groups; TK1 forms a group of its own; the other mammalian enzymes, dCK, dGK, TK2 and dNK, form a second and some viral kinases make a third group. Structural studies of the deoxyribonucleoside kinases has until recently only been reported for *Herpes simplex* 1 thymidine kinase (HSV1-TK) [6–10]. Now the three-dimensional (3D) structures of the cellular enzymes *Drosophila* dNK and human dGK have also been determined [11], and our main aim is to review the recent findings regarding the structure-function relationships of this class of enzymes.

Deoxyribonucleoside kinases are of medical interest as several human diseases are caused by defects in mitochondrial DNA (mtDNA) [12]. Such diseases are either maternally inherited or acquired as side effects of chemotherapy. Mitochondrial DNA replication occurs in all cells irrespective of their growth stages and requires several nuclear encoded enzymes. dGK is one of them and is involved in the regulation of the mitochondrial dGTP and dATP pools. The lack of dGK activity was recently linked to severe mtDNA depletion in several Druze families [13]. The children die at 1 to 4 months of age with liver failure and neurological abnormalities. No dGK enzyme was found in liver extracts from patients due to a deletion in the coding sequence. A similar mtDNA depletion syndrome but with severe muscle symptoms was the result of two types of point mutations in the TK2 protein, leading to low enzyme activity in extracts from muscle mitochondria [14]. Thus, information regarding the structure and function of TK2 and dGK can explain pathological processes involved in regulation of mitochondrial DNA synthesis.

The chemotherapeutic treatment of cancer and viral diseases depend largely on the action of deoxyribonucleoside kinases, which are key enzymes that catalyze the first step in the conversion of nontoxic nucleoside analogs to the corresponding nucleoside triphosphates. These then exhibit the toxic effect through initiation of apoptosis [15] or by inhibition of the DNA synthetic machinery.

Furthermore, deoxyribonucleoside kinases have lately become attractive candidates in suicide gene chemotherapy. The concept of this method is to introduce into the target cells a gene that encodes a nucleoside kinase. The expression of the kinase enhances the phosphorylation of nucleoside analogs, thereby increasing the sensitivity of the target cells to a chemotherapeutic agent with otherwise none or low toxicity. One gene, that of HSV1-TK, has already been used effectively in in vitro and in vivo models of human cancer [16, 17]. However, a deoxyribonucleoside kinase with a relaxed specificity and higher catalytic efficiency than HSV1-TK or the endogenous kinases would be an even more promising candidate. Therefore, the multisubstrate deoxyribonucleoside kinase dNK was recently proposed for use as a suicide gene [4] and has already been tested in human cancer cell models with encouraging results [18]. In addition, mutants of dNK with improved ability to mediate nucleoside-analog-induced cell killing have been developed [19].

Cytosolic thymidine kinase

TK1 (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) catalyzes the transfer of a γ-phosphate group from a nucleoside triphosphate to the 5′-hydroxyl group of thymidine. Among the deoxyribonucleoside kinases, TK1 has the most restricted substrate specificity and phosphorylates only thymidine and deoxyuridine. ATP and dATP are the preferred phosphate donors, but about 15–30% efficiency is obtained with (d)GTP, (d)CTP and (d)UTP, while dTTP is the feedback inhibitor [20, 21]. TK1 occurs as homodimers or homotetramers with a subunit size of 24 kDa [21–25].

The human TK1 gene was localized to the long arm of chromosome 17 several decades ago [26], and recently the position has been assigned to 17q25.2-25.3, within a region that harbors genes involved in sporadic breast cancer and ovarian tumorigenesis and other autosomal disorders [27, 28].

The human TK1 was cloned in 1984 by Bradshaw and Deininger and characterized by Flemington et al. [115, 116]. The entire gene spans 12.9 kb with seven exons. The complementary DNA (cDNA) is 1241 bp with an open reading frame of 702 bp, encoding a protein of 234 amino acids with a size of 25,505 Da.

TK1 or TK1-like genes seems to be widely distributed among living organisms. In the vertebrates human, mouse, hamster and chicken the TK1 sequences share a high homology. From a BLAST sequence similarity search (Swiss Institute of Bioinformatics) with the human TK1 amino acid sequence, homologous sequences were found to occur in nonvertebrates [*Caenorhabditis elegans, Dictyostelium discoideum* (slime mold)] and plants [*Oryza sativa* (rice), *Arabidopsis thaliana*], in many bacteria (e.g. *Escherichia coli, Bacillus subtilis,* several mycoplasma strains) and in a large number of viruses belonging to the pox family. However, TK1-like polypeptide sequences or activities are absent in insects *(Drosophila melanogaster)* and some unicellular fungi

(yeast). Although widely distributed, the physiological role of TK1 is still not known.

The expression of TK1 is cell cycle specific. TK1 activity is absent in resting cells, appears in late G1 cells, increases in S phase, coinciding with the increase in DNA synthesis and disappears during mitosis [29–31]. From a large number of studies it is clear that the characteristic cell-growth-correlated fluctuation of TK1 is partly due to S-phase-specific regulation of the TK1 promoter [31–37]. Furthermore, cyclin D1/CDK2 and the transcription factor E2F have been shown to be present in complexes binding to the mouse or human TK promoter [38, 39]. There are some indications of different or defect regulation of TK1 expression in cancer cells [40, 41].

Despite the strong regulation at the transcriptional level, posttranslational mechanisms seem to play an important role for the level of functional TK1 protein. There are several reports that human TK1 is phosphorylated [42, 43]. Phosphorylation took place at Ser13 in mitotically arrested cells; the responsible kinase was Cdc2 or Cdk2 kinase and the kinetic properties of phosphorylated TK1 were apparently unchanged [44]. This mitotic phosphorylation was shown to be blocked by overexpression of the cyclin-dependent kinase (CDK) inhibitor, $P21^{waf1}$. Thus, the C-terminal domain of P21 was found to interact with TK1 polypeptide in vivo and in vitro, but without any apparent effect on TK1 activity [45].

A posttranslational regulatory mechanism of significance for TK1 expression levels was associated with a sequence within the C-terminal 40 amino acids (but not the last 10 amino acids) of the TK1 polypeptide, signaling cell-cycle-specific degradation of the enzyme at the G2/M phase between metaphase and cytokinesis. Deletion of the 40 C-terminal amino acids abolished this G2/M-specific degradation and led to expression of the enzyme in quiescent cells [46, 47]. The C-terminal region in murine TK1 seemed to have the same effect on cell-cycle-specific degradation of the enzyme [48, 49]. It is worthwhile noticing that this C-terminal region is lacking in vaccinia TK, a thymidine kinase with close homology to mammalian TK1 (fig. 1) [49, 50].

On top of this comprehensive regulation, TK1 also appears to be regulated at the enzymatic level by ATP, thymidine and its own concentration. Exposure of pure TK1 from human lymphocytes to ATP induces a reversible enzyme concentration-dependent transition from

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                                        Region I    Region II
Human       MSCINLPTVLPGSPSKTRGQIQVILGPMFSGKSTELMRRVRRFQIAQYKC 50
Mouse       MSYINLPTVLPSSPSKTRGQIQVILGPMFSGKSTELMRRVRRFQIAQYKC 50
Ch. hamster MNYINLPTVLPGSPSKTRGQIQVILGPMFSGKSTELMRRVRRFQIAQNKC 50
Chicken     MNCLTVPGVHPGSPGRPRGQIQVIFGPMFSGKSTELMRRVRRFQLAQYRC 50
Vaccinia    MN---------------GGHIQLIIGPMFSGKSTELIRRVRRYQIAQYKC 35

Human       LVIKYAKDTRYSSS-FCTHDRNTMEALPACLLRDVAQEALGVAVIGIDEG 99
Mouse       LVIKYAKDTRYSNS-FSTHDRNTMDALPACMLRDVTQEALGVAVIGIDEG 99
Ch. hamster LVIKYAKDTRYSSS-FSTHDRNTMDALPACLLRDVAQEALGAAVIGIDEG 99
Chicken     LLVKYAKDTRYCTTGVSTHDRNTMEARPACALQDVYQEALGSAVIGIDEG 100
Vaccinia    VTIKYSNDNRYGTG-LWTHDKNNFEALEATKLCDVLESITDFSVIGIDEG 84

            Region III          Region IV
Human       QFFPDIVEFCEAMANAGKTVIVAALDGTFQRKPFGAILNLVPLAESVVKL 149
Mouse       QFFPDIVDFCEMMANEGKTVIVAALDGTFQRKAFGSILNLVPLAESVVKL 149
Ch. hamster QFFPDIVEFCEVMANAGKTVIVAALDGTFQRKAFGSILNLVPLAESVVKL 149
Chicken     QFFPDIVEFCEKMANTGKTVIVAALDGTFQRKAFGSILNLVPLAESVVKL 150
Vaccinia    QFFPDIVEFCERMANEGKIVIVAALDGTFQRKPFNNILNLIPLSEMVVKL 134

            Region IV     Region VI          Region VII
Human       TAVCMECFREAAYTKRLGTEKEVEVIGGADKYHSVCRLCYFKKASGQPAG 199
Mouse       TAVCMECFREAAYTKRLGLEKEVEVIGGADKYHSVCRLCYFKKSSAQTAG 199
Ch. hamster TAVCMECFREAAYTKRLGLEKEVEVIGGADKYHSVCRVCYFKKSSVQPAG 199
Chicken     NAVCMECYREASYTKRLGAEREVEVIGGADKYHSVCRACYFQKRP-QQLG 199
Vaccinia    TAVCMKCFKEASFSKRLGEETEIEIIGGNDMYQSVCRKCYIDS------- 177

Human       PDNKENCPVPGKPGEAVAARKLFAPQQILQCSPAN 234
Mouse       SDNK-NCLVLGQPGEALVVRKLFASQQVLQYNSAN 233
Ch. hamster PDNKENCPVLGQPGEASAVRKLFAPQQVLQHNSTN 234
Chicken     SENKENVPMGVKQLDMPASRKIFAS---------- 224
Vaccinia    ----------------------------------
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Figure 1. Sequence alignment of human TK1 and related enzymes. CLUSTAL X (1.8) multiple sequence alignment of TK1 homologous sequences. The conserved regions (underlined in human TK1) between the vertebrate and vaccinia virus kinases as outlined by Black and Hruby [50]. The sequences have SwissProt databank accession numbers P04183 (human), P04184 (mouse), P09768 (Chinese hamster), P04047 (Chicken) and P03297 (vaccinia virus).

a dimer with low thymidine affinity (K_m = 15 μM) to a tetramer with high thymidine affinity (K_m = 0.5 μM). The two TK1 forms have the same k_{cat} of about 4 s^{-1} [51]. The reaction kinetics of the dimer form appears to show negative cooperativity with thymidine, whereas the tetramer followed Michaelis-Menten kinetics [51]. According to the above-described fluctuation of the TK1 protein during the cell cycle, the concentration-dependent conversion from the low-affinity to the high-affinity form was suggested to serve as a fine tuner of TK1 activity during the cell cycle.

The substrate specificity of TK1 is very restricted, but some modifications are allowed at the 5 position of the pyrimidine ring, and the 3′ position of the deoxyribose [24, 52]. TK1 also phosphorylates several clinically important nucleoside analogs (table 1), e.g. 5-fluoro-2′-dideoxythymidine (FdU), the anti-human immunodeficiency virus (HIV) compounds, 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxy-2′,3′- didehydrothymidine (D4T) [53, 54]. AZT is an efficient substrate, while D4T is active at a level only 1–5% that of deoxythymidine (dT). The kinetic properties with AZT suggest that it is not a substrate for the low-affinity dimer form of the enzyme [55]. Several 5-substituted dUrd analogs are accepted by TK1, e.g. 5-fluoro, 5-chloro, 5-iodo, 5-bromo and 5-ethyl dUrd, while bulkier substitutions, such as 5-propenyl, 5-(2-chloroethyl) and 5-(2-bromovinyl), are not substrates [56, 57]. A number of large substitutions at the N3 position, e.g. *o*-carboranayl-alkyl dT, have been shown to be good substrates for TK1 [58].

A study using thymidine model compounds linked to affinity matrices showed strong binding of TK1 when thymidine was linked through the 5 position of the pyrimidine ring and the 5′ position of the sugar, but weak binding when thymidine was linked through the 3 and 3′ positions of the pyrimidine and sugar, respectively. This indicates that for binding to TK1, bulky substitutions at the 5 and 5′ positions are tolerated more than bulky substitutions at the 3 and 3′ positions of the pyrimidine deoxynucleoside [59].

dCK

dCK (NTP:deoxycytidine 5′-phosphotransferase, EC 2.7.1.74) phosphorylates pyrimidine and purine deoxyribonucleosides using UTP, ATP or other nucleoside triphosphates as phosphate donors. dCK is composed of two identical polypeptides of 261 amino acids [56, 60], and the enzymatic properties of recombinant human dCK are similar but not identical to dCK purified from human tissues [2, 52, 60–62].

The gene for human dCK is found on chromosome band 4q13.3-q21.1, and it is a single-copy gene of 34 kb [60, 63]. The expression of dCK messenger RNA (mRNA) is tissue specific and is high in lymphocytic tissues, intermediate in proliferating cells such as the colon mucosa and very low in differentiated tissues such as liver, muscle, kidney and pancreas [2, 60, 64]. The activity of the enzyme varied between 2- and 10-fold in extracts from different cells, while the mRNA levels are more constant, suggesting posttranscriptional regulation of dCK expression [65].

dCK has been described as a cytosolic enzyme in all the early studies, but Johansson et al. [66] demonstrated a nuclear localization signal in the N-terminal region of dCK. A nuclear localization of the enzyme when overexpressed in transfected cells was also observed. However, endogenous dCK was only found in the cytosol [67], and the role of the nuclear localization signal remains to be elucidated.

dCK shows a weak sequence similarity with HSV1-TK, as well as more than 30% identity with the TK2 and dGK sequences (fig. 2), and these enzymes constitute a family with many properties in common. The determination of the structure of human dGK and *Drosophila* dNK has provided a model for the structure of dCK as described below [11].

The reaction kinetics of dCK do not follow Michaelis-Menten kinetics but show negative cooperativity with the phosphate acceptors and donors, giving Hill coefficients below unity [61, 62]. The reaction mechanism was random bi-bi with ATP, while with UTP at low nucleoside concentrations it was ordered, with the donor appearing to bind before the acceptors [68]. Fluorescence quenching experiments showed that substrate binding induced conformational changes [69, 70], indicating that dCK exists in different conformational states with different affinities for the substrates. Deoxyribonucleoside triphosphates serving as feedback inhibitors (e.g. dCTP) were strong competitive inhibitors for the phosphate donors [71] and act as bisubstrate analogs as described below.

dCK phosphorylates deoxycytidine (dC) most efficiently but also deoxyadenosine (dA) and deoxyguanosine (dG), and in addition several pharmacologically used antiviral and cytostatic deoxyribonucleosides: β-L-2′3′-dideoxy-3′-thiacytidine (lamivudine), arabinosyl cytosine (cytosar), 2-chlorodeoxyadenosine (cladribine, CdA) and diflourodeoxycytidine (gemcitabine); 2-fluoro-arabinofuranosyl adenine (fludarabine, FAraA), 2′,3′-dideoxycytidine (zalcitabine, ddC) and arabinosyl adenine (vidarabine, AraA) [2, 57, 72–74]. The activities with the natural deoxyribonucleosides and some of these analogs are shown in table 1. The apparent K_m values for nucleoside substrates often decreased when UTP was used as phosphate donor instead of ATP [75]. UTP was also a preferred phosphate donor for dCK in phosphate transfer experiments when equimolar concentrations of ATP and UTP were present [76]. The mechanism for the influence of phosphate donors on the nucleoside specificity of dCK

remains to be explained but is most likely of importance for the in vivo function of the enzyme.

dCK accepts different enantiomeric forms of its substrates and in some cases show preferential phosphorylation of L-nucleosides [77–82]. dCK showed relatively high activity with L-OddC (β-L-1, 3-dioxolane-cytidine), and this L-nucleoside has antitumor activity [81, 82]. The fact that α-ddC is a more efficient substrate for dCK than β-ddC [77] demonstrates that dCK prefers nucleosides with the sugar in the S conformation (C2′-endo-C3′-exo), since α-ddC preferentially adopts this conformation. This was verified by determination of the structure of the bound nucleoside using nuclear magnetic resonance (NMR) methods with complexes of $^{13}C/^{2}H$ double-labeled dC and dA and dCK [83].

Synergistic effects of certain combinations of nucleosides, e.g. AraC and FArA [84], have been shown to at least in part be due to activation of dCK [85]. Incubation of cells with several of the nucleosides phosphorylated by dCK as well as with unrelated agents, such as the topoisomerase inhibitor etoposide, leads to higher dCK activity without a concomitant increase in dCK mRNA or protein levels [86]. The molecular nature of this posttranscriptional activation process is not defined, but the effect is of considerable clinic importance and could be utilized to improve anticancer and antiviral chemotherapy.

Mitochondrial thymidine kinase

TK2 (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21 (the EC number is the same as TK1) catalyzes the transfer of a γ-phosphate group from ATP to the 5′-hydroxyl group of deoxythymidine, the same substrate as for TK1. However, TK2 can also phosphorylate the other pyrimidines (see below). The level of TK2 is low compared with TK1 in proliferating cells, but in nonproliferating cells, it is the only pyrimidine deoxyribonucleoside phosphorylating enzyme.

The human TK2 gene has been localized to chromosome 16q22, and the expression of TK2 mRNA is complex and tissue specific. In most tissues, there are two transcripts of 4.0 and 2.2 kb, but in proliferating tissues, such as testis, ovary and thymus, two additional transcripts were found [87, 88]. The cloned cDNA sequences of human TK2 are incomplete as they do not include a mitochondrial targeting sequence. However, a mouse TK2 cDNA has been cloned, and the open reading frame codes for a polypeptide containing a mitochondrial targeting signal. In vitro translation and import experiments showed that the N-terminal sequence directs the import of precursor protein into isolated mitochondria [89]. A structural alignment of a truncated version of TK2 with the sequences of dGK, dCK and dNK is shown in figure 2.

Human TK2 utilizes dT, dC and deoxyuridine (dU) as its substrates but with different efficiency and kinetic mechanisms. TK2 phosphorylates dT with negative cooperativity, which means that the affinity for the substrate decreases with increasing substrate concentrations. However, the phosphorylation of dC and dU showed Michaelis-Menten kinetics. Both dTTP and dCTP inhibit the enzyme, and ATP and CTP can be used as phosphate donors [24, 88].

Thymidine analogs, such as AZT, arabinofuranosyl thymine (ara-T), 3′-fluoro-2′,3′-deoxythymidine (FLT) and ribothymidine could be phosphorylated, but with relatively low efficiency. A number of dU analogs could be phosphorylated by TK2, e.g. 5-halogen, amino, ethyl, 5-(2-bromovinyl), arabinosyl uracil (araU), 2′-difluoro-2′-deoxyuridine (dFdU) and 1-(2′-deoxy-2′-fluoro-1-beta-D-arabinofuranosyl)-5-iodouracil (FIAU) [24, 56, 88, 90]. Deoxycytidine analogs with 5-substitution, such as 5-(2-chloroethyl) and 5-(2-bromovinyl), showed activity with TK2, and modification of the sugar moiety, such as dFdC and AraC, was also acceptable but with low efficiency [56, 88]. Recent studies showed that TK2 is very efficiently inhibited by AraT or BVAraU (5-bromo-vinyl-deoxyuridine) analogs with bulky lipophilic substitutions at the 2′-OH [91]. The k_{cat}/K_m values for some of these nucleoside substrates are shown in table 1.

TK2, in contrast to TK1, has a relaxed stereoselectivity, i.e. both L-dT and L-dC are efficiently phosphorylated. Several other L-nucleosides such as L-BVDU, L-FMAU (2′-fluoro-5-methyl-β-L-arabinofuranosyluracil) and L-5-iodo-dU were also substrates for TK2 [78, 82, 88, 92].

The role of TK2 in mtDNA synthesis was recently clarified since it was shown that two substitutions (Ile181 to Asn and His90 to Asn) in the TK2 gene lead to partial enzyme deficiency and to mtDNA depletion myopath [14]. The children in two families had severe symptoms. Two were treated by external ventilation, and two died at 2 and 4 years of age. These results show that TK2 plays an essential role for the synthesis of mitochondrial DNA precursors and is involved in certain forms of mitochondrial diseases [89].

dGK

dGK (nucleoside triphosphate: deoxyguanosine 5′-phosphotransferase, EC 2.7.1.113) phosphorylates purine deoxyribonucleosides and their analogs, using a nucleoside triphosphate as phosphate donor. Like TK2, dGK is constitutively expressed, and similar activities are found in most tissues, including lymphoid tissues, spleen, skin, liver and brain [93]. The human dGK gene is localized to chromosome band 2q13, a region involved in translocations observed in some patients with lymphoproliferative disorders such as acute and chronic lymphatic leukemia [94].

Table 1. K_{cat}/K_m values for the cellular deoxyribonucleoside kinases (with 1–5 mM Mg-ATP)*.

	TK1	TK2	dNK	dGK	dCK
dT	8×10^6	9×10^5	2×10^7	$<1 \times 10^2$	2×10^2
AZT	3×10^6	8×10^4	4×10^3	$<1 \times 10^2$	$<1 \times 10^2$
FdU	2×10^6	3×10^5	2×10^7	$<1 \times 10^2$	$<1 \times 10^2$
dC	$<1 \times 10^2$	3×10^4	1×10^7	1×10^3	2×10^5
AraC	$<1 \times 10^2$	3×10^2	1×10^6	$<1 \times 10^2$	5×10^4
ddC	$<1 \times 10^2$	$<1 \times 10^2$	4×10^3	$<1 \times 10^2$	1×10^4
dFdC	$<1 \times 10^2$	2×10^4	nd	$<1 \times 10^2$	4×10^4
3TC	$<1 \times 10^2$	1×10^2	nd	$<1 \times 10^2$	1×10^5
dA	$<1 \times 10^2$	1×10^2	9×10^4	4×10^3	8×10^4
CdA	$<1 \times 10^2$	nd	nd	5×10^4	6×10^4
FAraA	$<1 \times 10^2$	$<1 \times 10^2$	nd	4×10^3	1×10^3
dG	$<1 \times 10^2$	$<1 \times 10^2$	2×10^4	6×10^4	6×10^4
AraG	$<1 \times 10^2$	$<1 \times 10^2$	nd	3×10^4	1×10^4

*The values in the table are calculated based on the low K_m values determined for TK2 and dCK, and the results are from [4, 5, 24, 54, 77, 89, 93, 96, 97]. nd, not determined.

Human dGK is a dimer of two identical subunits of 30 kDa, and dGK purified from various tissues/or species has similar properties but low specific activity, probably due to inactivation during purification. Recombinant dGK displays a much higher specific activity when purified in the presence of ATP and the detergent Triton X-100.

Several nucleoside triphosphates can act as phosphate donors, with ATP and UTP as the most efficient ones, while dATP and dGTP are feedback inhibitors. Natural purine deoxyribonucleosides, i.e. dG, dA and deoxyinosine (dI) are substrates for dGK (table 1). Nucleosides with modifications on either the sugar or the base were phosphorylated, e.g. antiviral and anticancer nucleoside analogs such as AraG (9-β-D-arabinofuranosylguanine), dFdG (2′,2′-difluorodeoxyguanosine), CdA, CAFdA (2-chloro-2′-arabinofluoro-2′-deoxyadenosine), AraA, F-AraA, and to a low extent ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)-guanine] and penciclovir [9-(2-hydroxy-1-hydroxymethyl-ethoxymethyl]-guanine [95, 96]. dGK also showed relaxed enantioselectivity, accepting both D- and L-enantiomers of β-dG with similar efficiencies but with low efficiency for α-L-dG, β-L-dA and β-L-dC [77, 97]. k_{cat}/K_m values for some of these nucleosides are shown in table 1.

A major 1.35-kb dGK mRNA transcript was found in most tissues by Northern blot analysis. The cDNA for human dGK codes for a 277-amino acid polypeptide with an N-terminal 17-amino acid sequence characteristic of a mitochondrial import signal (fig. 2), which is able to direct the precursor protein into the mitochondria. Biochemical studies of subcellular fractions as well as in situ immunohistochemical experiments using a dGK antibody clearly showed that the dGK protein is localized in the mitochondrial matrix [90, 98, 99], but unexpectedly dGK leaks out into the cytoplasm as efficiently as cytochrome c when cells undergo apoptosis [100]. The role of dGK in the apoptotic process remains to be determined, but dGK may be selectively transported into the cytosol to participate in generating apoptotic cofactors, such as dATP [15, 99].

The high activity of dCK in the activation of nucleoside analogs used in chemotherapy has prevented evaluation of the role of dGK for the cytotoxicity of nucleoside analogs. In cells with both dGK and dCK, dCK most likely is the main contributor in the activation of many nucleoside analogs, such as AraC, CdA and dFdC. However, in tissues that do not contain dCK, dGK is to a large extent responsible for the activation of purine nucleoside analogs.

D. melanogaster dNK

dNK (nucleoside triphosphate: deoxyribonucleoside 5′-phosphotransferease, the enzyme has recently been assigned its own EC number: EC 2.7.1.145). As outlined above, the mammalian kinases have been thoroughly studied, and until recently it was the general belief that TK1-, TK2-, dGK- and dCK-like enzymes were present in other eukaryotic organisms. Also, BLAST searches with these sequences found homologous sequences in plants and a wide variety of animals. Therefore, it was unexpected to find a different pattern in cells from *D. melanogaster*. Attempts to identify and clone TK1 from this organism failed, and instead a cellular approach based on separation and purification of the deoxyribonucleoside kinases from large quantities of cells was initiated. One of the initial steps in the purification procedure is separation of the four kinases by DEAE ion-exchange chromatography, but from cultured embryonic *D. melanogaster* cells, only one peak with deoxyribonucleoside kinase activity was obtained, containing all four activities [5, 101]. Further purification resulted in a single polypeptide with the ability to phosphorylate all four deoxyribonucleosides with high turnover (k_{cat}) as well as a broad variety of nucleoside analogs (see table 1). Furthermore, the turnover was higher than obtained with any of the known mammalian, viral and bacterial deoxyribonucleoside kinases. This broadly specific deoxyribonucleoside kinase was designated the name *Dm*-dNK, or dNK where N stands for any of the four deoxyribonucleosides, dT, dC, dA and dG.

dNK was cloned by two independent groups [4, 102]. When the entire genome of *D. melanogaster* was sequenced [103], it became clear that TK1, dCK and dGK were indeed absent in the fruitfly and that dNK was the only deoxyribonucleoside kinase present. The gene for dNK is located on *D. melanogaster* chromosome 3 at position 91D-91E, and the open reading frame is 750 pb encoding a polypeptide of 250 amino acids with a calculated

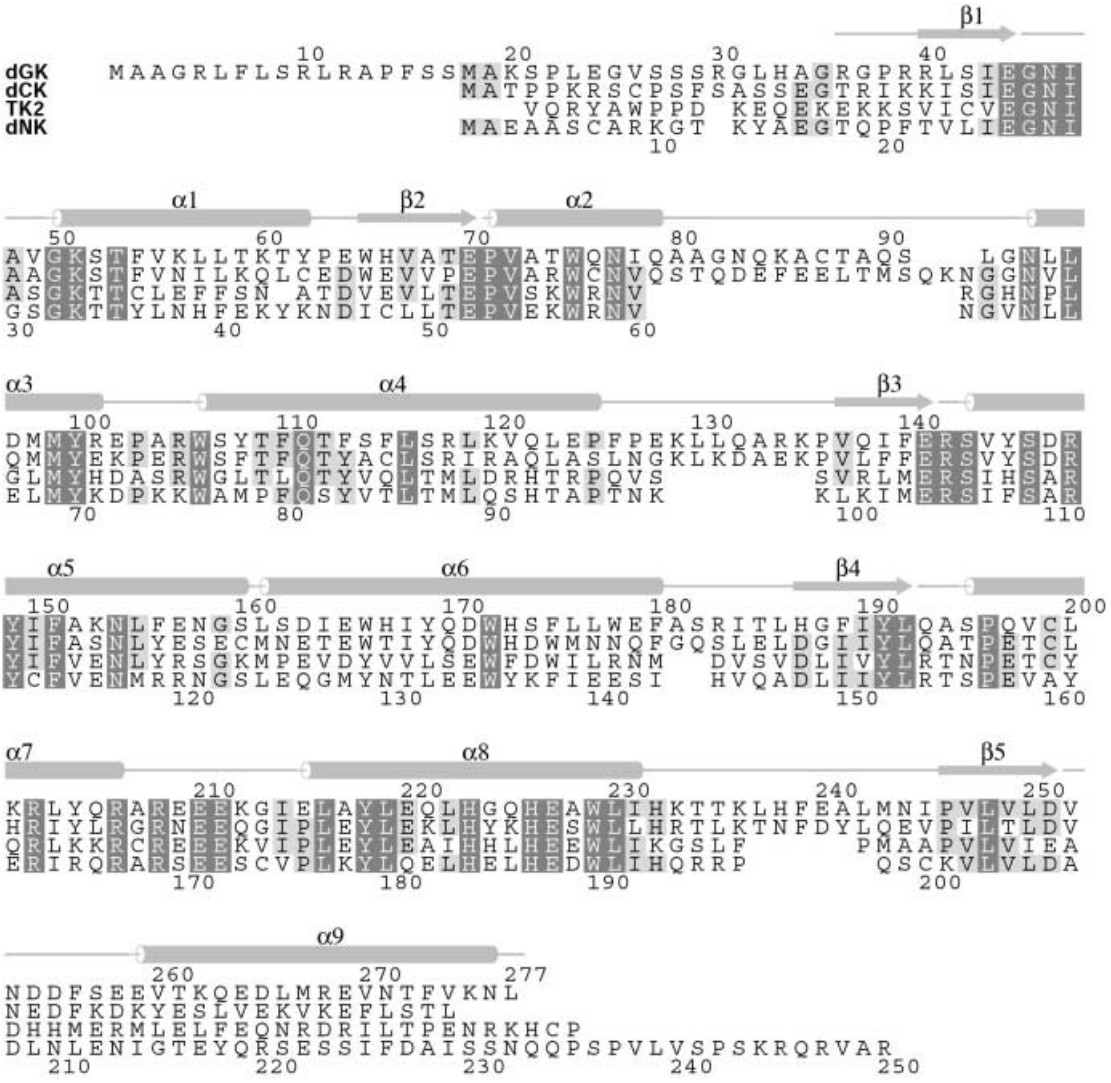

Figure 2. Structure based sequence alignment of the dNK-dCK-dGK-TK2 family. dGK, dCK and TK2 are the human sequences, whereas dNK is from *D. melanogaster.* Accession numbers are Q16854, P27707 and O00142 in the SwissProt databank and EMBL accession number Y18048.1, respectively. The numbers on top refer to the dGK sequences and the numbers below to the dNK sequence. The secondary structure for dGK, which is essentially the same for dNK, is given on top. Black fields represent residues identical in all four sequences, and shaded fields represent residues identical in three of them.

mass of 29,055 Da. The recombinant dNK has essentially the same substrate specificity and high turnover as the native enzyme. C-terminal deletion studies indicated the presence of an inhibitory sequence within the last 20 amino acids, since deletion of this part resulted in a mutant dNK with turnover more than twice as high as the wild type, but essentially the same substrate and analog specificity and K_m values [4]. A structural alignment with the sequences of TK2, dCK and dGK is shown in figure 2.

Whereas the kinetic reaction mechanisms with TK1, TK2 and dCK deviate from Michaelis-Menten kinetics, the *Drosophila* dNK reaction mechanism is straightforwardly hyperbolic, following a compulsory ordered steady-state reaction mechanism with formation of a ternary complex [5]. Initially, dNK was reported to be a monomer according to the elution profile on Superose 12 [5], but the three-dimensional structural analysis showed it to be a dimer [11], confirmed by recent results from gel filtration on Superdex 100 and native polyacrylamide gel electrophoresis (PAGE) [104].

The broad specificity of dNK towards the naturally occurring deoxyribonucleosides was reflected in a similarly broad specificity towards nucleoside analogs [4, 5]. dNK seems to combine the specificities of the mammalian kinases, as it is able to efficiently phosphorylate the TK2-specific analogs BVDU, FdUrd and Ara-T, and the dCK-specific Ara-C, and CdA. k_{cat}/K_m values for some of these nucleosides are shown in table 1.

Recently, it was shown that the multisubstrate deoxyribonucleoside kinase also exists in insects other than *D.*

melanogaster, since a kinase with the capacity to phosphorylate all four deoxyribonucleosides was cloned from *Bombyx mori* [104]. Like the fruitfly dNK, the silkworm dNK was a dimer with almost the same analog-phosphorylating register, although with a narrower specificity, as the purine ribonucleosides and dideoxyribosides were not phosphorylated. However, it did not show hyperbolic Michaelis-Menten substrate kinetics but exhibited positive cooperativity with all four natural deoxyribonucleosides. Among the deoxyribonucleoside kinases, this is the first reported case of positive cooperativity [104].

Whether a single dNK is a characteristic feature for insects remains to be elucidated. As for its physiological role, it was suggested that the different substrate specificities as well as the different feedback inhibition mode of dNK, which is only inhibited by dTTP as compared with TK2, which is inhibited both by dTTP and dCTP, may reflect substantially different cellular roles, where dNK in the insect cells may fulfill the function of TK1 [104].

The molecular basis for the specificity was analyzed by characterizing a number of mutant enzymes created by directed evolution [19]. Thirteen mutant dNKs were selected according to increased nucleoside analog sensitivity of TK-negative *E. coli* KY895 transfected with the mutant genes. The increased sensitivity of the *E. coli* cells to the drugs was supposedly due to a relative increase in the affinity of the mutant enzyme for the analogs. Surprisingly, the mutations were not located within the five regions highly conserved among the TK2-like deoxyribonucleoside kinases, but clustered around these. It was suggested that the changed sensitivity might be caused by structural changes in the neighborhood of the conserved motifs. The molecular background for the double mutant N45D/N64D giving the most pronounced increase in sensitivity was examined with the expressed and purified mutant enzyme. It was found that K_m increased and V_m decreased substantially with the natural substrates, but remained fairly unchanged with the analogs. Further, the IC_{50} (concentration giving 50% inhibition) value for dTTP feedback inhibition increased more than 20-fold when measured at 2–10 μM thymidine concentration. Whereas Asn45 is not conserved, Asn64 is highly conserved among the dNK/TK2 and dCK/dGK groups. Structural analyses showed Asn45 and Asn64 to be located far from the active site, offering little explanation for the profound effect on catalytic efficiency.

The relaxed specificity and high turnover of dNK makes it particularly interesting for biotechnological and medical applications. For instance, industrial large-scale production of dNTPs and analogs used for DNA synthetic reactions based on enzymatic synthesis of (deoxy)nucleoside monophosphates would give higher yield, and fewer toxic byproducts compared with the chemical processes.

The possibility of the existence of an insect-specific deoxyribonucleoside kinase and of interspecies differences in analog specificity opens the possibility for this enzyme as a target for the development of new and specific insecticides. For instance, BVDU was shown to prevent initiation of S phase in *D. melanogaster* cells, and to inhibit growth and development of *Spodoptera frugiperda* larvae to mature moths, but was only marginally cytostatic towards mammalian cells [105].

Additionally, dNK or dNK mutants with increased specificity towards certain analogs could be used as suicide genes in gene therapy. Zheng et al. have shown that two cancer cell lines transfected with the dNK wild-type gene had increased sensitivity towards a number of nucleoside analogs [18, 106, 107] . Using mutant dNKs, like the N45D/N64D [19], with high resistance to dTTP feedback inhibition and relatively increased analog specificity may improve the suicide gene therapy technique and make dNK mutants a promising alternative to HSV1-TK-based gene therapy, which is under investigation in clinical trials [17, 108].

Structures of deoxyribonucleoside kinases

Three-dimensional structures of deoxyribonucleoside kinases are available for human dGK, *Drosophila* dNK and *Herpes simplex* 1 thymidine kinase (table 2). The structure of the human dGK monomer has a central five-stranded parallel sheet with the strand order $\beta2$-$\beta3$-$\beta1$-$\beta4$-$\beta5$, and nine helices (fig. 3 a, b). The structure of dNK was determined on a C-terminal truncation mutant, where in addition to the 20 truncated residues, 16 more C-terminal residues were not visible in the electron density maps. In the dNK structure, this disordered part corresponds to the final helix of dGK. Otherwise, dNK contains the same secondary structure elements as dGK.

The core of HSV1-TK is similar to dNK and dGK with the same fold (fig. 3 c, d). The main difference between dNK/dGK and HSV1-TK (fig. 4) is the presence of an extra domain in HSV1-TK (between residues 197 and 198 in dNK; residues 236 and 237 in dGK), plus an extra C-terminal antiparallel β strand and an α helix, as compared with dNK and dGK. The extra domain is about 65 residues and is located on the opposite side of the subunit in relation to the active site. Furthermore, some of the helices are longer in HSV1-TK, particularly $\alpha3-\alpha4$.

The helices of dGK/dNK cover both sides of the sheet but extend further on the C-terminal side of the sheet to form the substrate sites and the subunit interaction area. The first and last strands are followed by a single helix, whereas the other three connections between the strands are by helix pairs in which the two helices are antiparallel to each other. Between $\beta2$ and $\beta3$, the first helix of the pair is broken into two helices, $\alpha2$ and $\alpha3$. Two sequence

Table 2. Crystallographic structures of deoxyribonucleoside kinases.

Enzyme	Space group	Resol. (Å)	R %	R_{free} %	Complex	PDB code	Reference
dGK	$P2_1$	2.8	26.5	28.0	ATP	1jag	11
dNK	$P2_12_12$	2.56	23.8	25.8	dC	1j90	11
dNK	$P2_1$	2.4	23.8	25.8	dTTP		K. Johansson, unpublished
HSV1-TK	$C222_1$	1.9	21.5			1e2h	120
HSV1-TK	$C222_1$	1.9	22.2		APS	1e2i	120
HSV1-TK	$C222_1$	2.5	21.2		dT	1e2j	120
HSV1-TK	$C222_1$	1.7	20.9		TMC	1e2k	121
HSV1-TK	$C222_1$	2.4	21.0		TMC	1e2l	121
HSV1-TK	$C222_1$	2.2			GA2	1ki2	8
HSV1-TK	$C222_1$	2.37			PE2	1ki3	8
HSV1-TK	$C222_1$	2.34	21.0		BTD	1ki4	8
HSV1-TK	$C222_1$	1.9			AC2	1ki5	6
HSV1-TK	$C222_1$	2.37			AHU	1ki6	8
HSV1-TK	$C222_1$	2.2	20.6		ID2	1ki7	8
HSV1-TK	$C222_1$	2.2	19.9		BVD	1ki8	8
HSV1-TK	$C222_1$	1.9	22.		BPG	1qhi	6
HSV1-TK	$I4_1$	2.75	18.2		ADP,TMP	1vtk	10
HSV1-TK	$I4_1$	2.8	17.5		ADP,THM	2vtk	10
HSV1-TK	$I4_1$	3.0			ADP,5IU	3vtk	10

motifs of the deoxyribonucleoside kinases are common to several nucleotide kinases of the same family, the P-loop and LID region. The conserved P-loop motif GXXXXGKS/TT is at the end of β1and at the turn between β1 and α1. The LID is an arginine-rich region with the consensus sequence RXXXRXRXXE located between α7 and α8 (residues 167–176 in dNK).

The structures of dNK and dGK are very similar, and 196 Cα atoms can be superimposed with a root mean square difference (r.m.s.d.) of 0.92 Å, which represents most parts of both structures. There are only a few residues at the termini plus residues 43–45, 98 and 196–198 of dNK, which are not included in this comparison. These positions represent differences in helix-strand loops between α1-β2, α4-β3 and α8-β5. The two latter are at positions with different chain lengths, dGK being the longer one. There are two additional places where dGK is longer. The main difference is the insertion of residues 79–90 (dGK numbering). HSV1-TK is a larger enzyme, and despite very low sequence identity (about 10%) the core of this enzyme is similar to dNK and dGK, with the same fold. This is reflected in that as many as 163 residues can be superimposed on dGK with Cα r.m.s.d. of 1.86 Å.

dGK, dNK and HSV1-TK are dimeric enzymes. The dimers are formed similarly in dNK and dGK (fig. 5), and the dimer interface is comprised of a hydrophobic area between helices α4 and α6 in each subunit, interacting to form a four-helix bundle. The central interaction area in dNK is composed of four aromatic, four aliphatic and four small residues from each subunit. Similarly, in dGK the dimer interaction contains several aromatic residues. The loop between residues 60 and 65 (dNK numbering), between α2 and α3, is an additional region seen to be part of the dimer contact area in dNK. Compared with dNK, dGK has an insertion of 12 extra amino acid residues in this region, which is partly disordered in dGK. The extra domain in HSV1-TK extends the subunit interaction area in that enzyme.

Substrate interactions in *Drosophila* dNK

The structure of dNK was determined on crystals that contained the substrate deoxycytidine. A sulfate ion was also present at the P-loop, mimicking the binding of one of the phosphate groups in the phosphate donor site. The long substrate cleft is situated perpendicular to the C-termini of the parallel β sheet (fig. 3a). The center of the cleft is located right after β3, where the main chain turns away at right angles from the sheet, and helix α5 continues roughly in the same direction as β3 but offset by about 10 Å. The conserved Glu104–Arg105 is located in the connection between β3 and α5. The part of the cleft where the substrate base binds is buried deep in the protein surrounded by helices α2, α3, α4 and α5.

The nucleoside is anchored to the enzyme by two hydrogen bonds to the base and four to the deoxyribose (fig. 6a). The cytosine base makes two hydrogen bonds to Gln81; the side-chain oxygen binds to the amino group in the 4-position, whereas the side-chain amino group binds to the nitrogen in the 3-position. The carbonyl oxygen in the 2-position is hydrogen bonded to two water molecules. One of the water molecules also binds to Tyr70, which is hydrogen bonded to the 3′-oxygen of the de-

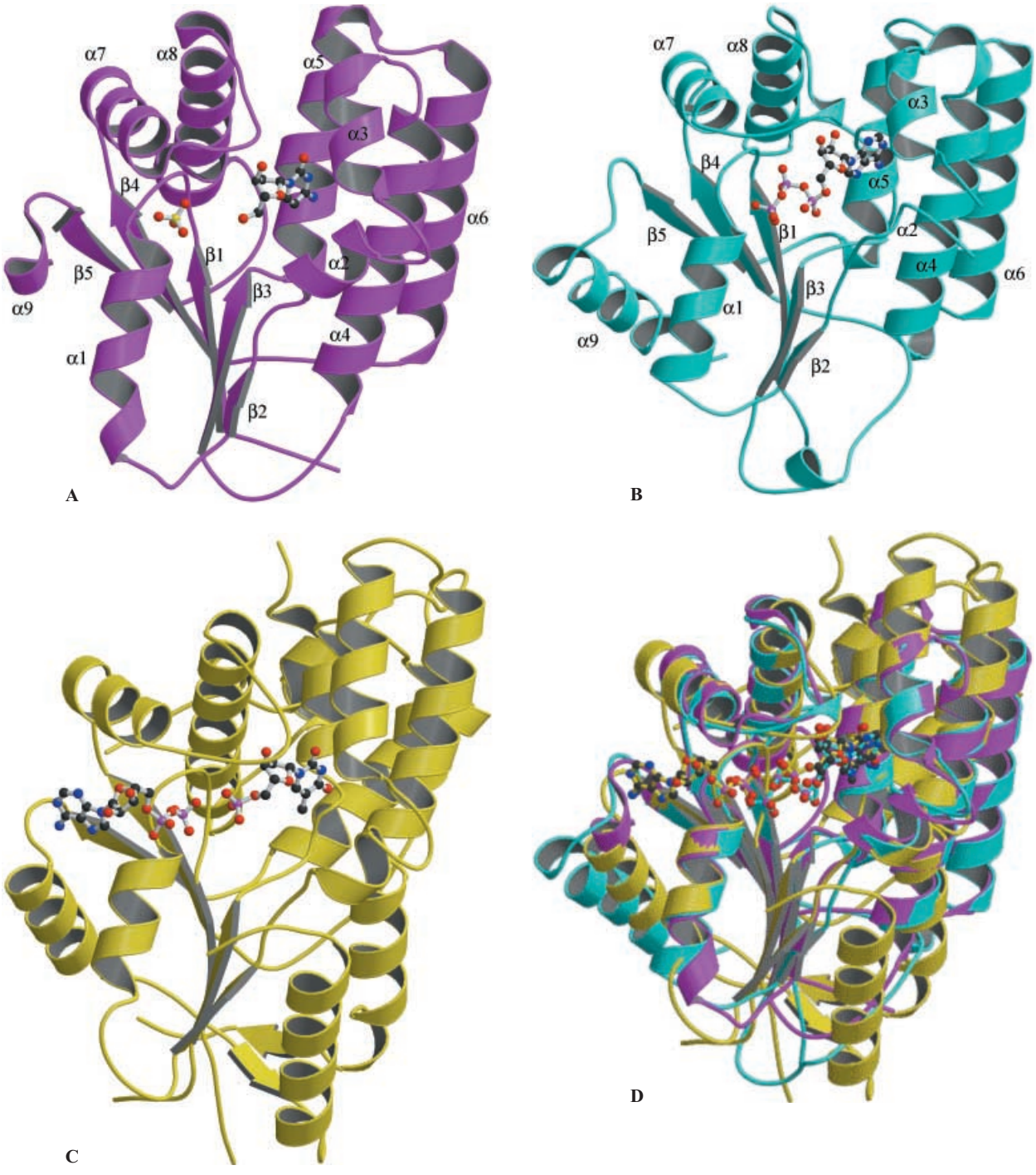

Figure 3. 3D structures of deoxyribonucleoside kinases. The three kinases have similar folds with a parallel five-stranded β sheet. (*A*). The subunit structure of dNK with β strands as arrows and α helices as ribbons, and bound deoxycytidine and sulfate ion (in ball-and-stick representation). (*B*). The subunit structure of dGK with bound ATP (in ball and stick representation). (*C*). The subunit structure of HSV1-TK with bound ADP and dTMP (in ball-and-stick representation). **(***D*). The subunit structures of dNK (magenta), dGK (green) and HSV1-TK (yellow), superimposed. The figures were made using MOLSCRIPT [122].

```
                              10         20         30         40         50         60
dGK           -----------MAAGRLFLSR LRAPFSSMAK SPLEGVSSSR GLHAGRGPRR LSIEGNIAVG KSTFVKLLTK
dNK           ---------------------- -------MAE AAASCARKGT KYAEGTQPFT VLIEGNIGSG KTTYLNHFEK
HSV1-TK       MASYPCHQHASAFDQAARSRG HNNRRTALRP RRQQKATEVR LEQKMPTLLR VYIDGPHGMG KTTTTQLLVA

                         70         80         90         100                    110

dGK           T--YPEWHVATE PVATWQNIQA AGNQKACTAQ SLGNLLDMMY REPA-------------RWSYTF
dNK           Y--KNDICLLTE PVEKWRNV-- ---------- NGVNLLELMY KDPK-------------KWAMPF
HSV1-TK       LGSRDDIVYVPE PMTYWRVLGA ---------- --SETIANIY TTQHRLDQGEISAGDAAVVMTSA

                    120        130               140        150        160        170

dGK           QTFSFLSRLK VQLEPFPEKL LQA--------RKPVQIF ERSVYSDRYI FAKNLFENGS LSDIEWHIYQ
dNK           QSYVTLTMLQ SHTAPTNK-- ------------KLKIM ERSIFSARYC FVENMRRNGS LEQGMYNTLE
HSV1-TK       QITMGMPYAV TDAVLAPHIG GEAGSSHAPPPALTLIF DRHPIAALLC YPAARYLMGS MTPQAVLAF-

                    180        190        200        210        220        230
dGK           DWHSFLLWEF ASRITLHGFI YLQASPQVCL KRLYQRAREE EKGIELAYLE QLHGQHEAWL IHKTTK
dNK           EWYKFIEESI --HVQADLII YLRTSPEVAY ERIRQRARSE ESCVPLKYLQ ELHELHEDWL IHQRRP
HSV1-TK       VALIP----- -PTLPGTNIV LGALPEDRHI DRLAKRQRPG ER-LDLAMLA AIRRVYGLLA NTVRYL

                                                                                  240
dGK           ------------------------------------------------------------------LHFE
dNK           ------------------------------------------------------------------
HSV1-TK       QGGGSWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAPNGDLYNVFAWALDVLAK

                    250        260        270     277
dGK           ALMNIPVLVL DVNDDFSEEV TKQEDLMREV NTFVKNL------------------------------
dNK           --QSCKVLVL DADLNLENIG TEYQRSESSI FDAISSNQQPSPVLVSPSKRQRVARLVSPSKRQRVAR
HSV1-TK       RLRPMHVFIL DYDQSPAGC- ---RDALLQL TSGMVQTHVTTPGSIPTICDLARTFAREMGEAN----
```

Figure 4. Structural alignment of deoxyribonucleoside kinase sequences. The human dGK, *D. melanogaster* dNK, and HSV1-TK are structurally aligned. Identical residues in all three structures are in red. The numbers are given for dGK.

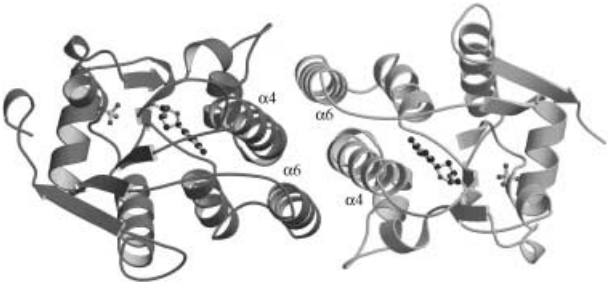

Figure 5. Dimer structure. *D. melanogaster* dNK dimer shown along the molecular twofold axis. The main interactions are between helices α4 and α6 of each subunit.

oxyribose. The second water molecule also binds to Gln81. On the opposite side of the base, there is a pocket with sufficient space for a methyl group at C5 for the binding of thymine. This pocket is occupied by two water molecules, one hydrogen bonded to Ser109 and the main-chain carbonyl of residue 106, while the second water molecule is bound to Glu52.

The base makes π-interaction with Phe114, with the ring planes roughly parallel. The other side of the base interacts end on with Trp57 and Phe80. Phe80 makes interactions to C2 and O2. Trp57 is in van der Waals contact with C5 and C6. Other van der Waals interactions are made by the base with Glu52, Met69, Tyr70, Val84, Met88, Ala110 and Met118 (fig. 6a).

The deoxyribose moiety is firmly anchored to the protein by two hydrogen bonds from Tyr70 and Glu172 to the 3′OH (fig. 6a). The 5′-oxygen is hydrogen bonded to Glu52, Arg169 and a water molecule. Glu52 is in turn hydrogen bonded to Arg105, whereas the water molecule is hydrogen bonded both to Arg105 and to the sulfate ion. C2′ is in contact with Ile29, Tyr70 and Ph114, O4′ with Leu66 and Trp67.

Nucleotide interactions in human dGK

When crystallizing dGK, the phosphate donor ATP was added (10 mM) since ATP is known to stabilize the enzyme. Unexpectedly, the ATP was found to bind not to the phosphate donor site, but with the adenine at the nucleoside substrate site (fig. 3b). The base and ribose of ATP are positioned in a manner similar to dC in dNK, while the phosphate groups occupy part of the phosphate-binding positions of the donor site. This binding of ATP at the nucleoside substrate site was not expected, but as will be discussed later, this mode of binding corresponds to that of a feedback inhibitor.

ATP binds at the substrate site, with Gln111 forming hydrogen bonds to N1 and N6 of the adenine ring and with a hydrogen bond from Arg118 to N7 (fig. 6b). Met99 and Asp147 are in the plane of the base, and Asp147 forms a hydrogen bond to Arg118. Phe110 and Phe151 are located on each side of the adenine base. The ring plane of

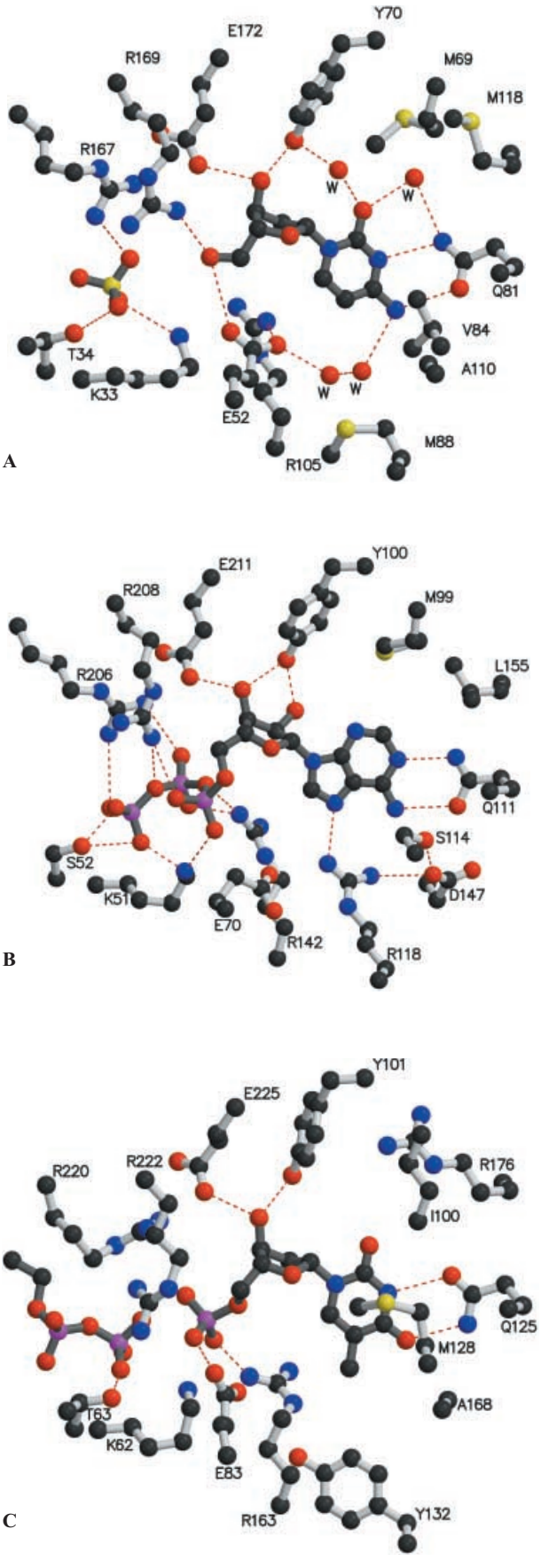

Figure 6. The nucleoside substrate site. (*A*) Binding of dC and the sulfate ion at the active site of dNK. Hydrogen bonds are red dotted lines. Water molecules are labeled W. (*B*) Figure with ATP interactions in dGK. Hydrogen bonds are red dotted lines. (*C*) Figure with ADP and dTMP interactions in HSV1-TK. Hydrogen bonds are red dotted lines.

Phe151 is parallel to the base plane, while Trp75 and Phe110 are at an angle to the base, as in dNK. The ribose O3′ atom is hydrogen bonded to Tyr100 and Glu211.

There are also a number of interactions with the triphosphate part of the ATP. The phosphates are bound by residues in the P-loop, Ala48, Lys51 and Ser52 and by residues Arg206 and Arg208 in the LID region, in addition to another residue, Arg142, present in the loop preceding helix α5. The way ATP is bound, the positions of the γ- and β-phosphates correspond approximately to the predicted positions of the β- and γ-phosphates of the phosphate donor, respectively (fig. 6b).

Phosphate donor site

The phosphate donor ATP binding site is characterized so far only in HSV1-TK (fig. 6c), and the donor site in dNK and dGK can be extrapolated from the structural similarity. The ATP site is formed between the N-terminus of α1, where the P-loop is located and the LID region that is a connecting loop between α7 and α8. The N-terminus of α9 should also be part of this binding site. In the structure of the truncated dNK protein, only a few residues of this helix are present. In the dNK structure, the phosphate donor site is occupied by a sulfate ion, which is bound to main-chain nitrogens and the side chains of Ser31, Lys33 and Thr34 of the P-loop as well as to Arg167 of the LID region (dNK numbering). Ser31 in dNK does not have any hydrogen-bonding counterpart in the other kinases of the family. The position of the P-loop and other parts of the phosphate donor site are very similar in nucleoside and nucleotide kinases, and the catalytic transfer of a phosphate group to the substrate thus occurs at very similar sites. The 5′OH of deoxycytidine in dNK superimposes closely to a phosphate oxygen of the nucleotide substrate in the monophosphate nucleotide kinases. A shift of the base and sugar of the nucleotide further into the substrate cleft achieves the shift from a nucleoside to a monophosphate nucleotide substrate.

Mechanism of action

The generally accepted mechanism for deoxyribonucleoside kinases (fig. 7) is that activation of the 5′-OH of the deoxyribose is required in order to make a nucleophilic attack on the γ-phosphate of the phosphate donor. The Glu carboxylate close to the 5′-OH at the active site of

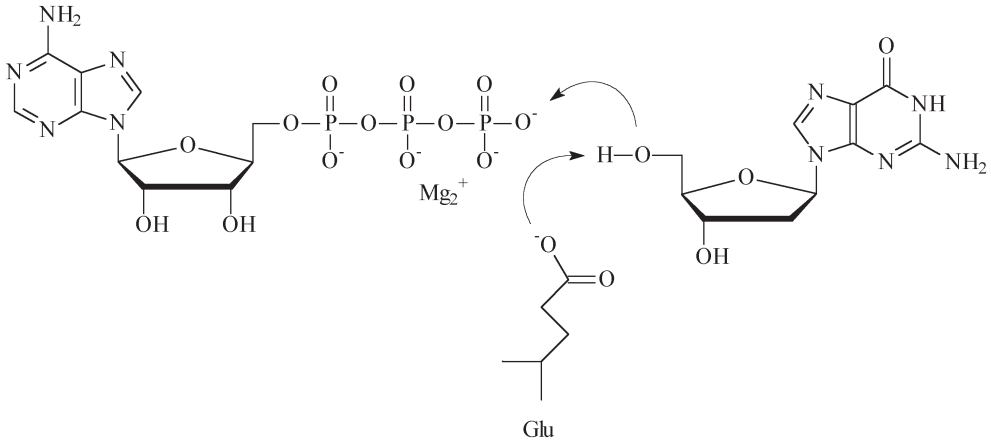

Figure 7. The mechanism of action of the deoxyribonucleoside kinases. The phosphate donor ATP is shown to the left and the substrate dG to the right. Glu is suggested to act as a general base and Mg^{2+} as a counter ion.

HSV1-TK was suggested to act as a base in the reaction that deprotonates the 5′-OH [10]. The close proximity of arginines should facilitate the deprotonation. In dNK and dGK, the presence of equivalent glutamic acids and arginines supports this mechanism. The activity of these kinases is dependent on Mg^{2+} ions, which for the related enzyme adenylate kinase is bound to the γ- and β-phosphates of the phosphate donor [109]. No structural information on Mg^{2+} binding to deoxyribonucleoside kinases is presently available.

Feedback inhibition

Several of the deoxyribonucleoside kinases are feedback regulated by the end-product deoxyribonucleoside triphosphates, and the best inhibitors are usually the triphosphates of the preferred substrates [110]. A mode of feedback inhibition was proposed for the inhibition of human deoxycytidine kinase by its feedback inhibitor dCTP [111]. On the basis of kinetic results, and on the strong and specific inhibition by dCTP, it was proposed that this end product functions as a bisubstrate analog, with its triphosphate group binding to the phosphate donor site of the enzyme and its deoxycytidine moiety overlapping and binding to the deoxyribonucleoside site in a highly specific manner.

For dNK, dTTP is a strong competitive inhibitor with respect to ATP, with K_i values in the nanomolar range [104] and a predominately uncompetitive inhibitor with respect to dT with inhibitory constants in the µM range [19]. In order to confirm that a true feedback inhibitor would bind in the same manner as was proposed from the dGK-ATP complex, the structure of a dNK complex with the feedback inhibitor dTTP was determined [K. Johansson et al., unpublished]. The deoxyribonucleoside part of dTTP has a very similar position at the active site, and the interactions of dT are practically identical to those of dC. Based on this dNK-dTTP complex structure, the hypothesis turned out to be accurate, and it can now be postulated that this mode of binding is that of a true feedback inhibitor.

Structural homology among the mammalian enzymes

The high sequence homology within the family (fig. 2) makes it possible to build homology models of other deoxyribonucleoside kinases. We have built models of the two other human enzymes in the family, TK2 and dCK. The sequence identity between TK1 and the others is too low to give a reliable homology model, though some structural features based on conserved sequence regions can be found and are discussed below.

TK2 model

The sequence identity between TK2 and dNK is 40% and there are no long insertions or deletions. The only difference, except for a C-terminal extension in dNK, is a one-residue deletion at the end of α1 and a one-residue insertion between α8 and β5 (fig. 2), both located at the surface far from the active site. Therefore, a reliable model of TK2 can be constructed based on the dNK structure. Such a model indicates that there are two differences in the substrate cleft: Leu116 (in TK2) in the position of Met118 (in dNK) is about 6 Å from the deoxycytidine base. Phe80, which is in van der Waals contact with the substrate base in dNK, is substituted by Leu in TK2. These substitutions make the substrate cleft of TK2 slightly larger.

TK2 is a strict pyrimidine nucleoside kinase, though the active site is strikingly similar to that of dNK, which is able to phosphorylate all four deoxyribonucleosides. However, the latter has much higher efficiency with pyrimidine substrates (table 1), which could explain the similarity of the active sites between the two enzymes.

dCK model

The human dCK amino acid sequence is about 45% identical to that of human dGK. Since dGK is a mitochondrial protein, there is a signal peptide at the N-terminus, and the sequence is 17 residues longer here than for the cytosolic dCK. Besides this and that dCK is three residues shorter at the C-terminus, there is only one difference in chain length: a three-residue insertion between α2 and α3 in dCK. This part of the structure is a disordered loop close to the subunit interaction area in dGK. Modeling of dCK based on dGK shows that there is only one side-chain difference in the substrate pocket. There is an Ala in dCK instead of Ser114 in dGK about 4 Å from the purine substrate. Ser114 in dGK is hydrogen bonded to Asp147, which is hydrogen bonded to Arg118, Tyr169 and Gln111. Ala in the corresponding position in dCK should make the substrate site slightly larger close to the N6 of an adenine.

The large similarities between the active site of dCK and dGK are consistent with their good activity with purine substrates. However, dGK phosphorylates only purines, whereas dCK preferentially phosphorylates dC as well as the purines dG and dA. It is not obvious why a change of Ser to Ala should give rise to a high dC activity.

Structural aspects TK1

There are no reports available about the 3D structure of TK1 or polypeptides with close homology. The only strong sequence similarity between the TK1 and the other cellular deoxyribonucleoside kinases is the P-loop, which probably has a similar position after the first β strand. All other assignments are speculative, since there are no significant sequence similarities with the other kinases of the family.

The P-loop has the sequence GPMFSGKST in human TK1 with the conserved glycines and a lysine followed by two Ser/Thr, which is a consensus sequence for the deoxyribonucleoside kinases as well as other related kinases. TK1 has a Ser preceding the second conserved glycine, like dNK and TK2. This Ser forms a hydrogen bond to the sulfate ion at the phosphate donor site. By aligning vertebrate TK1 with the vaccinia TK, seven homology regions were identified (fig. 1) [50]. Using vaccinia virus TK, Black and Hruby identified the ATP magnesium binding sites to the P-loop [50].

Mutational studies indicated that the aspartate in the vaccinia TK sequence VIGI**D**EG is involved in magnesium binding, but the D82N mutant bound ATP as the wild-type enzyme (fig. 1) [112]. Mutational studies showed that Gln in the sequence DGTF**Q**RH is involved in feedback inhibition with dTTP [113].

The reversible transition of human TK1 between a dimer and a tetramer was shown to be associated with residues in a highly conserved segment of the sequence [114]. The published sequence [115, 116] shows a methionine at site 106, but sequence analysis of 21 cDNAs or genomic DNAs from apparantly normal cells and malignant cell lines revealed a valine at site 106 in human TK1 similar to the other mammalian TKs and vaccinia TK [114]. Met^{106}TK1 was a permanent tetramer with high thymidine affinity (K_m = 0.5 µM), whereas Val^{106}TK1 behaved as the native TK1 from human lymphocytes as described above [51, 114]. In a structural prediction of the TKs using JPred [117], the consensus secondary structure of the sequence DIV^{106} EFCEAMA is an α helix, which is supported by a study of the secondary structure of vaccinia TK by circular dichroism (CD) spectroscopy of synthetic peptides [118]. The region around V106 suggests it forms an amphiphatic helix, which would facilitate possible subunit interaction in this region.

Substrate specificity for the deoxyribonucleoside kinases

The substrate clefts in dNK, dGK and HSV1-TK have some conserved features. They all have a glutamine residue that makes edge-on hydrogen bonds to the base. A glutamine has the advantage that it can easily turn its side chain amide group and adapt to the type of base that binds and form hydrogen bonds to any base. All structures also have an aromatic residue that stacks to the base, with a Trp always present on the opposite side, whereas a third stacking residue in this area varies. It is a Phe in dNK and dGK, whereas it is an Ala in HSV1-TK. A conserved feature is also a Tyr-Glu pair, which forms hydrogen bonds to the 3′OH of the substrate deoxyribose. In addition, a Glu and an Arg are always found close to the 5′OH (see table 3).

The types of side chains that make contacts in the plane of the base in the different deoxyribonucleoside kinases vary, and the differences in specificity for the different bases should largely depend on these differences (fig. 6). It appears that dNK, which has the broadest substrate specificity, has a large unspecific substrate cleft, while dGK, which has a stronger preference for purines, has a tighter binding pocket.

The catalytic efficiency of dNK is higher for dC and dT compared with TK1, TK2 and dCK (table 1). The active site cavity in dNK is indeed large enough to accommodate the different substrates, but there are no obvious features that can explain the differences in catalytic efficiency. Only the conserved glutamine residue, Gln81, which can bind to the C4 and C6 of purines, and C2 and C4 of pyrimidines, is in direct contact with the substrate base. This fact makes it hard to formulate a hypothesis about what determines the substrate specificity of the *Drosophila* enzyme. Factors not directly involved in binding of the substrate should be examined further to understand the details of the enhanced catalytic efficiency for the pyrimidine substrates.

Table 3. Substrate interactions.[a]

Interactions	dNK	TK2	dGK	dCK	HSV1-TK
Hydrogen bonds to substrate base	Gln 81	Gln 79	Gln 111	Gln 97	Gln 125
In plane	Glu 52	Glu 50	Glu 70	Glu 53	Glu 83
	Met 69	Met 67	Met 99	Met 85	Ile100
	Tyr 70	Tyr 68	Tyr 100	Tyr 86	Tyr 101
	Val 84	Val 82	Ser 114	Ala 100	Met 128
	Met 88	Met 86	Arg 118	Arg 104	Tyr 132
	Ala 110	Ala 108	Asp 147	Asp 133	Ala 168
	Met 118	Leu 116	Leu 155	Leu 141	Arg 176
Stacking	Trp 57	Trp 55	Trp 75	Trp 58	Trp 88
	Phe 80	Leu 78	Phe 110	Phe 96	Ala 124
	Phe 114	Phe 112	Phe 151	Phe 137	Tyr 172
	Val 84	Val 82	Ser 114	Ala 100	Met 128[b]
O3′ hydrogen bonds	Tyr 70	Tyr 68	Tyr 100	Tyr 86	Tyr 101
	Glu 172	Glu 170	Glu 211	Glu 197	Glu 225
O3′ surroundings	Ile 29	Ile 28	Ile 47	Ile 30	His 58
	Leu 66	Leu 64	Leu 96	Leu 82	Ile 97
O5′	Glu 52	Glu 50	Glu 70	Glu 53	Glu 83
	Arg 105	Arg 103	Arg 142	Arg 128	Arg 163
	Arg 169	Arg 167	Arg 208	Arg 194	Arg 222
Phosphates	Ser 31	Ser 30	Val 49	Ala 32	Met 60
	Lys 33	Lys 32	Lys 51	Lys 34	Lys 62
	Thr 34	Thr 33	Ser 52	Ser 35	Thr 63
	Thr 35	Thr 34	Thr 53	Thr 36	Thr 64
	Arg 167	Arg 165	Arg 206	Arg 192	Arg 220
	Arg 169	Arg 167	Arg 208	Arg 194	Arg 222

[a] Amino acid residues at equivalent positions in the enzymes are listed in each row.
[b] The position of the side chain of Met 128 is the same as Phe 80, Leu 78, Phe 110 and Phe 96 and forms stacking interactions to the base in HSV1-TK, but the position of Ca of Met 128 is equivalent to that of Val 84, Val 82, Ser 114 and Ala 100.

dGK selectively phosphorylates purine deoxyribonucleosides with the highest efficiency for dG. Thus, the active site of dGK should have some additional residues accounting for this specificity. Indeed, Arg118 gives additional binding and stabilization of dG and dI, but not of the other deoxyribonucleosides. The arginine would make good interactions both with the ring N7 and carbonyl O6 present in dG (fig. 6b). These interactions are similar to the most common interactions of protein-DNA interactions, where Arg frequently binds guanine with hydrogen bonds to N7–O6 ([119], and references therein). Adenine has an amino group at the 6-position, which makes the binding less favorable. Arg118 is held tightly in place by Asp147, which in turn is positioned by hydrogen bonds to Gln111, Tyr169 and Ser114. Furthermore, residues Arg118-Asp147-Gln111 form a triad that can form more favorable interactions with each other in the presence of dG than with dA. The presence of the arginine is not suitable for binding dT with a methyl group in this position, which explains the low activity with this substrate. Additional contacts with the substrate are equivalent to those in dNK.

The position around the 2′ position of the substrate sugar is crowded. In dNK, there are two residues closer than 4 Å to the C2′ position, Ile29 and Tyr70, making ribose less favorable than deoxyribose. However, there is no strong discrimination against the 2′OH group, but the differences are apparently sufficient to give a significant kinetic difference. Binding of ATP at high concentrations in the dGK crystal also demonstrates that no dramatic structural changes have to take place in order to bind a ribonucleotide in the substrate binding site. It should be noted that dNK has indeed some activity with ribonucleosides, best with pyrimidines – 16% with C, 6% with U – and about 1% with the purines, when compared with dT (100%) [4].

The topography of the active site clefts for dNK and dGK (fig. 8) can now be explored to design new analogs and explain the specificities of existing analogs. In dNK, HSV1-TK and most likely also in TK2, there is a pocket at the 5-position of the pyrimidine ring suitable for the methyl group in dT, as well as analogs with substitutions at this position such as the antiherpetic drug BVDU. BVDU is a good substrate for dNK, TK2 and HSV1-TK, but not for dGK and dCK. This pocket is missing in dGK and also in the dCK model based on dGK. Instead, on the opposite side of the base, there is a cleft in dGK suitable for the amino group at 2-position of dG. This cleft is present also in dNK and HSV1-TK and is slightly larger.

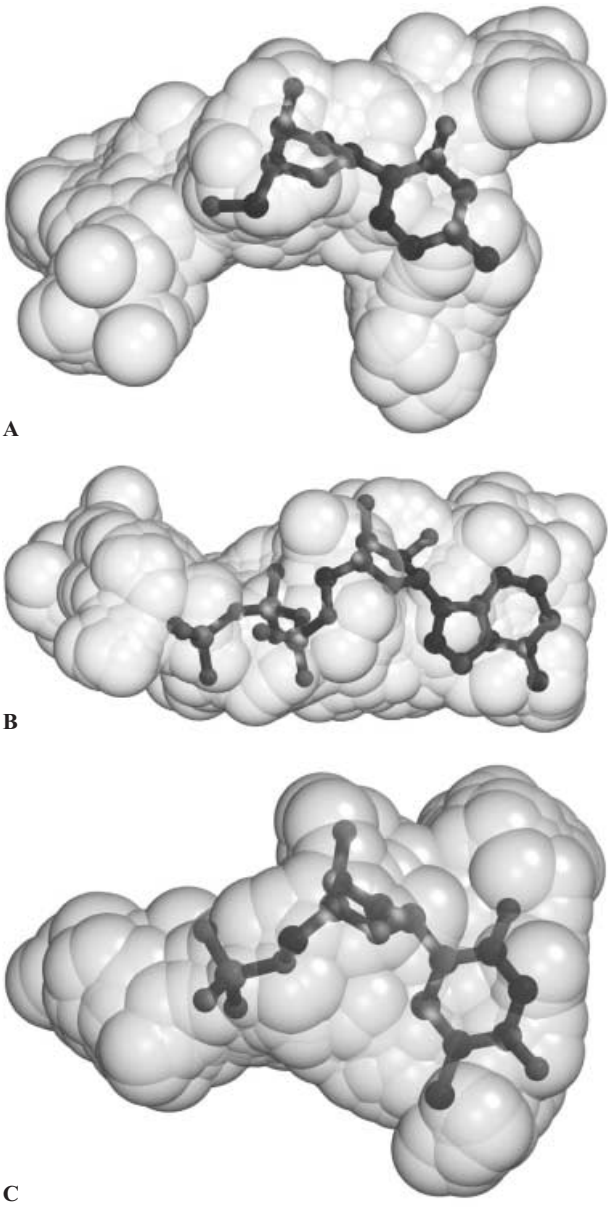

Figure 8. The substrate binding pocket in deoxyribonucleoside kinases. The substrate pockets of dNK, dGK and HSV1-TK with bound substrates. The figure shows the accessible surface (made using the program Acsite, Devapriya Choudhury, Department of Molecular Biology, Swedish University of Agricultural Sciences). (*A*) The substrate cleft in dNK with bound dC. **(***B*) The substrate site in dGK with bound ATP. (*C*) The substrate site in HSV1-TK with bound dTMP.

Conclusions

Since the genes for a large number of deoxyribonucleoside kinases are known and several of the gene products have been or can be expressed in recombinant form, detailed studies of the structure and function of this enzyme family is now possible. The initial steps in this line of research have been completed as outlined in this review, and these results put us in a position to use molecular modeling techniques, cocrystallization methods and in vitro mutagenesis to define the detailed structure-function relationships within this family. Studies with medically important target enzymes such as the deoxyribonucleoside kinases described here, but also others found in many pathogenic microorganisms, may aid future rational drug design of nucleoside analogs. Although the active site structures most likely are similar within this family, detailed knowledge about the differences can be used to design selective and active chemotherapeutic nucleoside analogs and inhibitors. In case of TK1, the 3D structure is needed to define the differences and similarities to the other kinases. Also, several biological questions, such as the transcriptional and posttranscriptional regulation of deoxyribonucleoside kinases in different tissues and cells, as well as in normal, malignant and infected cells, remain to be clarified. All these exciting tasks eagerly await future research efforts.

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