A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines

Wolfgang Knecht^{1,2}, Michael P.B. Sandrini¹,
Kenth Johansson³, Hans Eklund³,
Birgitte Munch-Petersen⁴ and Jure Piškur¹

¹Section of Molecular Microbiology, BioCentrum-DTU, Building 301, Technical University of Denmark, DK-2800 Lyngby, ⁴Department of Life Sciences and Chemistry, Roskilde University, DK-4000 Roskilde, Denmark and ³Department of Molecular Biology, Swedish University of Agricultural Sciences, Box 590, Biomedical Center, S-751 24 Uppsala, Sweden

²Corresponding author
e-mail: wolfgang.knecht@biocentrum.dtu.dk

In mammals, the four native deoxyribonucleosides are phosphorylated to the corresponding monophosphates by four deoxyribonucleoside kinases, which have specialized substrate specificities. These four enzymes are likely to originate from a common progenitor kinase. Insects appear to have only one multisubstrate deoxyribonucleoside kinase (dNK, EC 2.7.1.145), which prefers pyrimidine nucleosides, but can also phosphorylate purine substrates. When the structures of the human deoxyguanosine kinase (dGK, EC 2.7.1.113) and the dNK from *Drosophila melanogaster* were compared, a limited number of amino acid residues were identified and proposed to be responsible for the substrate specificity. Three of these key residues in *Drosophila* dNK were then mutagenized and the mutant enzymes were characterized regarding their ability to phosphorylate native deoxyribonucleosides and nucleoside analogs. The mutations converted the dNK substrate specificity from predominantly pyrimidine specific into purine specific. A similar scenario could have been followed during the evolution of kinases. Upon gene duplication of the progenitor kinase, only a limited number of single amino acid changes has taken place in each copy and resulted in substrate-specialized enzymes.

Keywords: deoxyribonucleoside kinases/directed mutagenesis/enzyme specificity/nucleoside analogs/protein evolution

Introduction

Deoxyribonucleoside kinases catalyze the phosphorylation of deoxyribonucleosides to the corresponding deoxyribonucleoside monophosphates (dNMPs). They are the key enzymes in the salvage of deoxyribonucleosides originating from extra- or intracellular breakdown of DNA. Subsequently, dNMPs are phosphorylated into diphosphates (dNDPs) and triphosphates (dNTPs), which are the precursors of DNA. Deoxyribonucleoside kinases play a key role in the chemotherapeutic treatment of cancer and viral diseases, as they catalyze the first, and

often rate-limiting step of nucleoside analog activation by phosphorylation (Arnér and Eriksson, 1995). Native and genetically engineered deoxyribonucleoside kinases from different organisms are also attractive candidates for use in cancer gene therapy as suicide enzymes (Christians *et al.*, 1999; Kokoris *et al.*, 1999; Knecht *et al.*, 2000a; Zheng *et al.*, 2000). The basic concept here is to transduce cancer or virus-infected cells with a gene encoding a deoxyribonucleoside kinase and subsequently expose them to a nucleoside analog. The activation of the nucleoside analog to a cytotoxic or antiviral compound is potentiated by the transduced kinase (Culver *et al.*, 1992; Guettari *et al.*, 1997). A role for suicide genes has also been proposed for the purging of selected cell populations in therapies that involve bone marrow transplantation (Bonini *et al.*, 1997; Lal *et al.*, 2000). Additional biotechnological applications of deoxyribonucleoside kinases lie in the enzymatic synthesis of dNTPs and their analogs (Knecht *et al.*, 2000a; Munch-Petersen *et al.*, 2000).

The number and specificities of different deoxyribonucleoside kinases vary from organism to organism. Mammals have four deoxyribonucleoside kinases with overlapping specificities (Arnér and Eriksson, 1995). Thymidine kinase 1 (TK1, EC 2.7.1.21) phosphorylates only thymidine (Thd) and deoxyuridine (dUrd). The pyrimidine-specific thymidine kinase 2 (TK2, EC 2.7.1.21) phosphorylates Thd and dUrd, but also deoxycytidine (dCyd). Deoxyguanosine kinase (dGK, EC 2.7.1.113) phosphorylates deoxyadenosine (dAdo) and deoxyguanosine (dGuo), while the deoxycytidine kinase (dCK, EC 2.7.1.74) phosphorylates dAdo, dGuo and dCyd (Arnér and Eriksson, 1995; Hatziz *et al.*, 1998; Jüllig and Eriksson, 2000; Wang and Eriksson, 2000). Mutations in the genes of the mitochondrially located human TK2 and dGK have recently been linked to inherited and severe mitochondrial DNA depletion syndromes (Mandel *et al.*, 2001; Saada *et al.*, 2001).

Both the fruit fly *Drosophila melanogaster* and the silk worm *Bombyx mori* possess a multisubstrate deoxyribonucleoside kinase (dNK, EC 2.7.1.145) capable of phosphorylating all four natural deoxyribonucleosides (Munch-Petersen *et al.*, 1998, 2000; Knecht *et al.*, 2002). However, from the evolutionary point of view, these dNKs are related to the mammalian TK2 enzymes (Munch-Petersen *et al.*, 2000; Knecht *et al.*, 2002). It is likely that all four mammalian deoxyribonucleoside kinases and the insect dNKs have originated from a common progenitor kinase. Apparently, the first gene duplication generated the progenitor of TK1- and TK2-like kinases, including dNKs and dCK/dGK-like kinases (Knecht *et al.*, 2002). The progenitor of TK2-, dCK/dGK-like kinases, could have a broad substrate specificity, which during evolution has been narrowed down in different kinases, leading to the modern enzymes.

The structural basis for the different substrate specificities of the eukaryotic dNKs was unknown until recently, when a directed evolution study on the *D.melanogaster* dNK (Dm-dNK) indicated residues involved in the specificity and regulation of the fruit fly dNK (Knecht *et al.*, 2000a). Mutants of Dm-dNK generated by hypermutagenesis were isolated based on their ability to increase the sensitivity of transformed TK-deficient *Escherichia coli* KY895 to various nucleoside analogs. One mutant, MuC, harboring the V84A mutation, altered the sensitivity of transformed KY895 selectively for dCyd analogs, such as dideoxycytidine (ddCyd) and 1- β -D-arabinofuranosylcytosine (AraC). It is noticeable that the valine at position 84 in Dm-dNK is conserved in all eukaryotic TK2-like kinases and that all eukaryotic dCKs have an alanine at this position (Figure 1). The recently solved crystal structure of

Dm-dNK and human dGK additionally contributed to an understanding of the structure–function relationship (Johansson *et al.*, 2001), and showed that Val84 in Dm-dNK is one of the amino acids surrounding the deoxyribonucleoside base-binding pocket in Dm-dNK. Both Dm-dNK, as a representative of the TK2-like kinases, and human dGK, as a representative of the dCK/dGK-like kinases, had a similar overall fold (Johansson *et al.*, 2001). Despite the low sequence identity of herpes simplex virus thymidine kinase (HSV1-TK, EC 2.7.1.21) (Figure 1) to Dm-dNK or human dGK, the core structure of HSV1-TK has a similar fold to that of Dm-dNK and human dGK (Johansson *et al.*, 2001). The deoxyribonucleoside base-binding pocket in the structures is flat, and differences in the specificities for various nucleoside bases should depend largely on the amino acids surrounding this substrate cleft. The multisubstrate kinase Dm-dNK has a large substrate cleft, dominated by hydrophobic residues, while the purine deoxyribonucleoside-specific human dGK has a tighter binding pocket containing charged residues which could support specific hydrogen bonds to purine substrates (Johansson *et al.*, 2001). Dm-dNK has an extended cleft at the 5-position of the substrate pyrimidine ring, lined by Val84, Met88 and Ala110 (Figure 2A and B). This empty cavity, which is absent in human dGK (Johansson *et al.*, 2001), would be necessary to accommodate the methyl group of Thd.

In the present study, we attempted to modify a dNK into an enzyme with high substrate specificity, and possibly mimic the evolutionary processes that generated the modern enzymes. Dm-dNK, with its uniquely broad substrate acceptance, was used as a model for TK2- and dCK/dGK-like kinases, and three amino acids lining the nucleoside base-binding pocket of Dm-dNK were mutated into the corresponding residues of dCK/dGK-like kinases (Figure 1). The results obtained are also valuable for the rational design of new, more efficient antiviral and anti-cancer drugs; they can help to understand the structural

		V84	M88		A110
TK2-like kinases	Dm-dNK	81	QSYVLTLMQ	104	ERSIFGARYCF
	Bm-dNK	68	QSYVSLTMLD	91	ERSLFGARYCF
	Xen-PyK	130	QTYVQLTMLD	153	ERSIYSARYYF
	human-TK2	79	QTYVQLTMLD	102	ERSIHGARYYF
	mouse-TK2	115	QTYVQLTMLD	138	ERSIYSARYYF
dCK/dGK-like kinases	human-dCK	97	QTYVCLSRIR	127	ERSVYSDRYIF
	mouse-dCK	97	QSYVCLSRIR	127	ERSVYSDRYIF
	rat-dCK	97	QSYVCLSRIR	127	ERSVYSDRYIF
	human-dGK	111	QTFSEFLSLK	141	ERSVYSDRYIF
	mouse-dGK	111	QTLSEFLSLK	141	ERSVYSDRYIF
	HSV1-TK	125	QITGMPMAV	162	DRHPIAALLCY

Fig. 1. Two partial multiple alignments of the predicted amino acid sequences of 10 eukaryotic deoxyribonucleoside kinases and HSV1-TK. Bm-dNK, *Bombyx mori* multisubstrate deoxyribonucleoside kinase; Xen-PyK, *Xenopus laevis* pyrimidine deoxyribonucleoside kinase. The positions in Dm-dNK where mutations were introduced are marked with arrows. The corresponding amino acids in the other kinases are marked with a black background. The conserved residues in all kinases are marked as light gray, and the conserved residues in the eukaryotic deoxyribonucleoside kinases, but not in HSV1-TK, are marked as dark gray.

Fig. 2. Crystal structure of the Dm-dNK subunit with dCyd at the active site (Johansson *et al.*, 2001). (A) The residues which have been mutated are shown in ball and stick representation. ATP (in white) is modeled from the HSV1-TK to show the location of the phosphate donor (Wild *et al.*, 1997; pdb code 1vtk). (B) Close-up view of dCyd bound at the substrate site showing the positions of residues Val84, Met88 and Ala110. Red dotted lines represent hydrogen bonds. (C) Model of the mutations V84S, M88R and A110D based on the crystal structures of dNK and dGK. The conformation of these side chains are as in dGK (Johansson *et al.*, 2001).

basis for mutations that are found in mutated human deoxyribonucleoside kinase genes and in the development of novel mutant enzymes for suicide gene therapy and biotechnical synthesis of dNTPs and their analogs.

Results

V84A

We initiated our present investigation by examining the significance of the V84A mutation and additionally mutated Val84 into the corresponding amino acids typical for dGKs and HSV1-TK, which are S and M, respectively (Figure 1). The kinetic parameters of the purified mutant

Dm-dNK-V84A, together with the other mutant Dm-dNKs, are shown in Table I. In Table II, the relative catalytic efficiencies of the Dm-dNKs are compared with other eukaryotic deoxyribonucleoside kinases. The decrease in the catalytic efficiency for Thd is 10-fold larger than for dCyd, and there is only a marginal decrease in the efficiencies for dAdo and dGuo compared with the wild-type Dm-dNK. Apparently, the V84A change discriminates against Thd. A reason for this may be that a valine in this position should make favorable interactions with the methyl group of Thd. Another interesting aspect is that Dm-dNK-V84A increased the sensitivity of *E. coli* KY895 transformed by this mutant towards ddC and AraC

Table I. Kinetic parameters of wild-type and mutant Dm-dNKs

	Dm-dNK	K_m (μM)	$V_{\max}$ (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	Decrease in k_{cat}/K_m of the mutants compared with k_{cat}/K_m of Dm-dNK (fold)
Thd	V84A	11.3	5.7	2.97	2.6×10^5	46.2
	V84S	24.7	1.6	0.83	3.3×10^4	363
	V84M	829	0.07	0.037	84	142 857
	M88R	237	0.25	0.13	548	21 897
	A110D	64	0.67	0.35	5468	2194
	M88R/A110D	2425	0.065	0.034	14	857 142
	V84A/M88R/A110D	1512	0.22	0.11	72	166 667
	V84S/M88R/A110D ^a	6560	0.44	0.23	35	342 857
	wild-type ^b	1.2	29.5	14.2	1.2×10^7	1
	V84A	2.5	8.5	4.4	1.76×10^6	4.1
dCyd	V84S	12.9	1.6	0.83	6.4×10^4	112.5
	V84M	674	3.4	1.8	2670	2697
	M88R	1039	14.5	7.5	7218	997
	A110D	91	0.53	0.27	2967	2426
	M88R/A110D	584	6.7	3.5	5993	1201
	V84A/M88R/A110D	627	3.7	1.94	2615	2753
	V84S/M88R/A110D	2162	3.1	1.62	749	9613
	wild-type ^b	2.3	34.2	16.5	7.2×10^6	1
	V84A ^c	69.7	7.1	3.7	5.3×10^4	1.7
	V84S	149.7	2.1	1.1	7348	12.5
dAdo	V84M	851.5	2.6	1.36	1597	57.6
	M88R	2088	3.6	1.88	900	102
	A110D	238.7	0.16	0.084	352	261
	M88R/A110D	359.2	13.2	6.9	1.9×10^4	4.8
	V84A/M88R/A110D	153	6.6	3.5	2.3×10^4	4
	V84S/M88R/A110D	388	6.1	3.2	8247	11.2
	wild-type ^b	225	42.7	20.6	9.2×10^4	1
	V84A	146.6	5.4	2.8	1.9×10^4	1.2
	V84S	375	1.22	0.64	1706	13.5
	V84M	1911	0.425	0.22	115	200
dGuo	M88R ^d	644.7	5.2	2.7	4187	5.5
	A110D	256.9	0.14	0.073	284	81
	M88R/A110D	549	7.05	3.7	6740	3.4
	V84A/M88R/A110D ^e	40.4	3.3	1.73	4.3×10^4	0.53
	V84S/M88R/A110D	159.2	8.8	4.6	2.9×10^4	0.79
	wild-type ^b	665	31.3	15.1	2.3×10^4	1
	V84A	292	1.8	0.93	3185	1.2
	wild-type ^g	1124	8.6	4.2	3737	1
	AraC ^f	33.5	4.9	2.53	7.6×10^4	1.6
	wild type	24.3	5.6	2.9	1.2×10^5	1

The k_{cat} values were calculated using the equation $V_{\max} = k_{\text{cat}} \times [\text{E}]$, where $[\text{E}]$ = total enzyme concentration and is based on one active site/monomer.

^a K_m , and therefore also $V_{\max}$, can only be considered as a rough estimate, because K_m lies well above the tested Thd concentrations (up to 2500 μM).

^bData from Munch-Petersen *et al.* (2000).

^cThe data are best described by the Hill equation with: $K_{0.5} = 58.7 \mu\text{M}$, $V_{\max} = 6.5 \text{ U/mg}$, $h = 1.7$.

^dThe data are best described by the Hill equation with: $K_{0.5} = 239.5 \mu\text{M}$, $V_{\max} = 3.05 \text{ U/mg}$, $h = 2.4$.

^eThe data are best described by the Hill equation with: $K_{0.5} = 36.3 \mu\text{M}$, $V_{\max} = 3 \text{ U/mg}$, $h = 1.5$.

^f K_m and $V_{\max}$ were determined by the spectrophotometric assay.

^gData from Knecht *et al.* (2000a).

Table II. Comparison of the relative catalytic efficiencies k_{cat}/K_m for the Dm-dNK mutants and the four mammalian deoxyribonucleoside kinases

	Thd	dCyd	dAdo	dGuo
Dm-dNK ^a	100	60	0.8	0.19
V84A	2.2 (14.7)	14.7 (100)	0.44 (3)	0.16 (1.1)
V84S	0.28 (51.6)	0.53 (100)	0.06 (11.5)	0.014 (2.7)
V84M	0.0007 (3.1)	0.022 (100)	0.013 (59.8)	0.001 (4.3)
M88R	0.0046 (7.6)	0.06 (100)	0.0075 (12.5)	0.035 (58)
A110D	0.46 (100)	0.025 (54.3)	0.003 (6.4)	0.0024 (5)
M88R/A110D	0.00012 (0.07)	0.05 (31.5)	0.16 (100)	0.056 (35.5)
V84A/M88R/A110D	0.0006 (0.17)	0.022 (6.1)	0.192 (53.5)	0.36 (100)
V84S/M88R/A110D	0.0003 (0.12)	0.0062 (2.6)	0.069 (28.4)	0.24 (100)
TK1 ^b	66.7 (100)	— ^c	— ^c	— ^c
TK2 ^b	0.158 (100)	0.092 (58.2)	— ^c	— ^c
dCK ^c	— ^c	0.61 (100)	0.022 (3.6)	0.0225 (3.7)
dGK ^d	— ^c	— ^c	0.00019 (8.3)	0.0023 (100)

The catalytic efficiency for wild-type Dm-dNK and Thd was set to 100%. The relative catalytic efficiencies for each enzyme are given in parentheses, and the substrate having the highest efficiency was set to 100% for each enzyme.

^aData from Munch-Petersen *et al.* (2000).

^bData from Munch-Petersen *et al.* (1991).

^cData from Bohman and Eriksson (1998).

^dData from Wang *et al.* (1993).

^e ‘—’ no activity was reported with the corresponding substrate.

(Knecht *et al.*, 2000a). The kinetic values of Dm-dNK-V84A for ddC and AraC are given in Table I. Because in a cell, the simultaneous presence of all four nucleoside substrates should be considered, the difference in efficiencies between two enzymes can be compared using the equation $[k_{\text{cat}}/K_m (\text{nucleoside analog})]/[k_{\text{cat}}/K_m (\text{dAdo}) + k_{\text{cat}}/K_m (\text{dCyd}) + k_{\text{cat}}/K_m (\text{dGuo}) + k_{\text{cat}}/K_m (\text{Thd}) + k_{\text{cat}}/K_m (\text{nucleoside analog})]$ (Kokoris *et al.*, 1999). For Dm-dNK-V84A, this equation predicts an increase in catalytic efficiency for the phosphorylation of ddC by 8-fold and for AraC by 6-fold. These values correlate quite well with the previously observed changes in the LD₁₀₀ for transformed KY895 (Knecht *et al.*, 2000a).

V84S

A serine at position 84 in Dm-dNK decreased the catalytic efficiency for all deoxyribonucleosides, but more for pyrimidine deoxyribonucleosides (by 8- and 27-fold more for dCyd and Thd, respectively) than for purine deoxyribonucleosides. Apparently, the serine at this position, as found in dGKs, helps to discriminate against pyrimidine deoxyribonucleosides. The reason for this is not obvious from available structural information (Johansson *et al.*, 2001). The position of a serine in an equivalent position in dGK is ~4 Å from the 4-position of a pyrimidine substrate.

V84M

A methionine at position 84 in Dm-dNK caused a striking decrease in the catalytic efficiency for Thd when compared with the other substrates. In addition, the catalytic efficiencies for dCyd, dAdo and dGuo were strongly decreased as compared with Dm-dNK-V84A and Dm-dNK-V84S. The V84M mutant was investigated because the corresponding residue in HSV1-TK is a methionine. However, the stacking Phe80 of Dm-dNK is missing in HSV1-TK where the corresponding residue is an alanine. The side chain of methionine in HSV1-TK, corresponding

to V84 in Dm-dNK, therefore substitutes for the stacking phenylalanine. In the V84M mutant, there is not enough space for the methionine side chain since Phe80 is present. The methionine side chain would then have to occupy another position and thereby disturb the binding of the substrate base, particularly for Thd, which has a methyl group in this position.

Creating a polar face of the substrate pocket

dCK/dGK-like kinases have a conserved arginine corresponding to Met88 in Dm-dNK (Figure 1). In the human dGK, this Arg118 was previously postulated to be responsible for the high selectivity for purine deoxyribonucleosides and in particular the high efficiency with dGuo (Johansson *et al.*, 2001). Because Arg118 is held tightly in place by Asp147 in the structure of human dGK, we made three mutants of Dm-dNK: Dm-dNK-M88R, Dm-dNK-A110D and the double mutant Dm-dNK-M88R/A110D.

In Dm-dNK-M88R, a dramatic decrease in efficiency was observed for Thd as compared with the other substrates. In contrast to Dm-dNK-V84A and Dm-dNK-V84S, Dm-dNK-M88R phosphorylated the purine deoxyribonucleoside with higher efficiency than Thd. Nevertheless, dCyd remained the preferred substrate. Position 88 is in the center of the binding site for the methyl group of Thd, which explains the drastic effect on Thd.

The change of alanine to aspartate at position 110 in Dm-dNK caused a decrease in efficiency with all substrates, ~10-fold more for pyrimidine than purine deoxyribonucleosides. However, Thd remained the preferred substrate, as with wild-type Dm-dNK. The reason for this is not obvious from available structural information (Johansson *et al.*, 2001). The position of an aspartate in an equivalent position in dGK is ~4 Å from the 6-position of an adenine (4-position of a pyrimidine).

The double mutant Dm-dNK-M88R/A110D showed the strongest decrease in efficiency for Thd, >800 000-fold, while the decrease in efficiency for dCyd was just 1200-

fold and only ~4-fold for dAdo and dGuo. The reason for the strong effect of the double mutation for Thd is that space for the Thd methyl group is occupied by the arginine and the introduction of unfavorable charges in the methyl site. Thereby, dAdo became the most efficiently converted substrate, followed by dGuo. Compared with the single mutations M88R and A110D, a double mutation is apparently necessary to change the pyrimidine-preferring Dm-dNK into a purine-preferring deoxyribonucleoside kinase. In dGK (Johansson *et al.*, 2001), there are hydrogen bonds present between the corresponding residues (Arg118 and Asp147; Figure 1), which prevent them from moving away from this site and thereby promoting binding of purines.

An even more pronounced preference for purine deoxyribonucleosides was achieved when the V84A or V84S mutations were combined with the M88R/A110D double mutation. Both triple mutants Dm-dNK-V84A/M88R/A110D and Dm-dNK-V84S/M88R/A110D showed strongly decreased efficiency for phosphorylation of Thd and the strongest decrease in efficiency for phosphorylation of dCyd. For both triple mutants, dGuo became the preferred substrate, with an improvement in efficiency even compared with Dm-dNK. Dm-dNK-V84A/M88R/A110D phosphorylated dCyd and dAdo with higher efficiency than Dm-dNK-V84S/M88R/A110D. This supports the suggestion by Johansson *et al.* (2001) that the alanine/serine difference, corresponding to the Val84 position of Dm-dNK, between dCKs and dGKs might be associated with the ability of dCKs to phosphorylate dCyd with higher efficiency than dGKs.

In three cases, as marked in Table I, the kinetic data could be described better by using the Hill equation instead of the Michaelis–Menten equation but, for the sake of simplicity (Wang and Eriksson, 2000), all data were compared on the basis of the Michaelis–Menten equation.

Phosphorylation of deoxyribonucleoside analogs

The relative phosphorylation of substrates other than those in Table I was also investigated. The results, as compared with Dm-dNK, are given in Table III. While Dm-dNK cannot phosphorylate dideoxyguanosine (ddGuo), all mutants having the V84A, V84S or M88R mutation could accept ddG as a substrate. The Thd nucleoside analogs dideoxythymidine (ddT) and 3'-azido-2',3'-dideoxythymidine (AZT) were not accepted by the double or triple mutants, while 1- β -D-arabinofuranosylthymine (AraT) was not accepted as a substrate for the triple mutants. In contrast, the dAdo analogs 9- β -D-arabinofuranosyladenosine (AraA), 2-fluoroadenine 9- β -D-arabinofuranoside (F-AraA) and 2-chloro-deoxyadenosine (CdA) became relatively better substrates for the double and triple mutants when compared with Thd. Surprisingly, this was also observed for the Thd analog 5-bromo-vinyl-deoxyuridine (BVDU). Dm-dNK-V84A/M88R/A110D, Dm-dNK-V84A and Dm-dNK-V84S could phosphorylate the Thd analog 2',3'-didehydro-3'-deoxythymidine (D4T). Dm-dNK-V84A/M88R/A110D was unique in its ability to convert the sugar 2'-deoxyribose (2-dR) alone, without any base attached. Furthermore, a number of mutations, V84S, V84M, M88R and V84A/M88R/A110D, introduced into Dm-dNK the ability to phosphorylate acyclic

guanosine (ACV) and/or ganciclovir (GCV), which cannot be phosphorylated by the wild-type enzyme.

Discussion

Specificity for deoxyribonucleosides

The three-dimensional structure and the catalytic machinery of three deoxyribonucleoside kinases, namely Dm-dNK, human dGK and HSV1-TK, have recently been shown to be very similar. Therefore, the specificity of the enzymes for different natural deoxyribonucleosides, as well as for medically important nucleoside analogs, should depend on only a few amino acid differences in the substrate pocket (Johansson *et al.*, 2001). Our data clearly show that a change in the amino acids surrounding this cavity severely changes the specificity of Dm-dNK (Table I). In general, all tested mutations increased the relative catalytic efficiency for purine deoxyribonucleosides in each mutant when compared with Thd (Table II). By mimicking the situation in eukaryotic dCK/dGK-like kinases in this area, we could convert Dm-dNK from a pyrimidine-preferring deoxyribonucleoside kinase into an almost exclusively purine-preferring deoxyribonucleoside kinase (Tables I and II). In particular, the triple mutant Dm-dNK-V84S/M88R/A110D (Figure 2C) closely resembles dGK, but with considerably higher absolute catalytic efficiency (Table II). When comparing Dm-dNK, Dm-dNK-V84A and Dm-dNK-V84S, as well as the two triple mutants, it is obvious that an alanine at position 84 favors the relative phosphorylation of dCyd. The triple mutant Dm-dNK-V84S/M88R/A110D, mimicking the nucleoside-binding site of dGK, also showed substrate preferences similar to dGK. The triple mutant Dm-dNK-V84A/M88R/A110D, mimicking the amino acid residues found in dCK, showed higher catalytic efficiencies for dAdo and dCyd than the dGK-mimicking triple mutant (Table II). Modeling of dCK based on the three-dimensional structure of dGK revealed that there is no difference in the side chains lining the substrate pocket, except for an alanine instead of a serine at the site corresponding to Val84 in Dm-dNK (Johansson *et al.*, 2001). Our data support the hypothesis that this 'exchange' might be the basis for the high capacity for dCyd phosphorylation observed in dCK. The reason for this is not obvious from available structural information. The position of an alanine should be ~5 Å from the 4-position of dCyd. Why the extra space at this position created by the smaller alanine should favor dCyd as a substrate is a mystery at present. This suggests that other factors must also be crucial for the superior dCyd phosphorylation ability of dCK when compared with dGK. None of the mutations created here have been found as yet in mutated human deoxyribonucleoside kinase genes (Mandel *et al.*, 2001; Saada *et al.*, 2001).

Phosphorylation of deoxyribonucleoside analogs

When comparing the relative phosphorylation of Thd nucleoside analogs and Thd by wild-type Dm-dNK, we would expect the same tendency of a decrease in specific activity as displayed for Thd by the mutant proteins (Table III). This was also observed for all analogs having a methyl substitution at the 5-position of the Thd base, except D4T, for which we could measure phosphorylation with three mutants but not with the wild-type enzyme.

Table III. Substrate specificities of wild-type and mutant Dm-dNKs

Substrate (500 μ M)	Dm-dNK	V84A	V84S	V84M	M88R	A110D	M88R/A110D	V84A/M88R/ A110D	V84S/M88R/ A110D
Thd	100 ^a	100 (19)	100 (5.1)	100 (0.09)	100 (0.58)	100 (2)	100 (0.04)	100 (0.19)	100 (0.11)
dUrd	113 $\pm$ 2 ^a	107 $\pm$ 8 (20.3)	90.6 $\pm$ 12.4 (4.6)	249 $\pm$ 133 (0.22)	810 $\pm$ 5.1 (4.7)	96.5 $\pm$ 4.1 (1.9)	334 $\pm$ 20.5 (0.13)	191 $\pm$ 17 (0.36)	206 $\pm$ 6.6 (0.23)
FdUrd	92 $\pm$ 11 ^a	97.6 $\pm$ 2.6 (18.5)	93.4 $\pm$ 1.8 (4.8)	126 $\pm$ 19.5 (0.11)	353 $\pm$ 6.5 (2)	89.7 $\pm$ 5.1 (1.8)	69 $\pm$ 7.8 (0.028)	34.1 $\pm$ 3.8 (0.065)	76.3 $\pm$ 24.6 (0.084)
ddThd	4.4 $\pm$ 0.4 ^a	5.2 $\pm$ 0.5 (0.99)	3.1 $\pm$ 0.9 (0.16)	0.4 $\pm$ 0.1 (0.00036)	n.d.	1.4 $\pm$ 1.2 (0.028)	n.d.	n.d.	n.d.
ddCyd	— ^b	— ^b	3.2 $\pm$ 0.2 (0.16)	0.5 $\pm$ 0.1 (0.00045)	n.d.	0.9 $\pm$ 0.2 (0.018)	n.d.	n.d.	n.d.
ddAdo	0.9 $\pm$ 0.3 ^a	0.5 $\pm$ 0.1 (0.095)	1 $\pm$ 0.2 (0.051)	0.5 $\pm$ 0.1 (0.00045)	n.d.	n.d.	13.4 $\pm$ 5.9 (0.0054)	22 $\pm$ 11 (0.042)	15.5 $\pm$ 13.1 (0.017)
ddGuo	n.d. ^a	0.6 $\pm$ 0.1 (0.11)	0.3 $\pm$ 0.1 (0.015)	n.d.	7.5 $\pm$ 2.2 (0.044)	n.d.	19.2 $\pm$ 8.4 (0.0077)	12.8 $\pm$ 10.2 (0.024)	18.4 $\pm$ 10.7 (0.02)
Cyd	16.2 $\pm$ 2 ^a	23.9 $\pm$ 1 (4.5)	4.8 $\pm$ 0.5 (0.24)	n.d.	1.5 $\pm$ 0.7 (0.0087)	4.9 $\pm$ 0.2 (0.098)	n.d.	5 $\pm$ 1.7 (0.0095)	n.d.
Urd	6.5 $\pm$ 0.5 ^a	3.5 $\pm$ 0.3 (0.67)	0.6 $\pm$ 0.5 (0.031)	0.2 $\pm$ 0.1 (0.00018)	3.1 $\pm$ 2.2 (0.018)	3.3 $\pm$ 3.1 (0.066)	n.d.	n.d.	n.d.
Ado	0.8 $\pm$ 0.3 ^a	1 $\pm$ 0.2 (0.19)	0.4 $\pm$ 0.3 (0.02)	1 $\pm$ 0.4 (0.0009)	0.8 $\pm$ 0.6 (0.0046)	n.d.	28.1 $\pm$ 13.1 (0.011)	35.2 $\pm$ 9.8 (0.067)	19.5 $\pm$ 8.5 (0.021)
Guo	1.2 $\pm$ 0.2 ^a	n.d.	n.d.	n.d.	3.6 $\pm$ 0.4 (0.021)	n.d.	n.d.	19.3 $\pm$ 1.9 (0.037)	23.5 $\pm$ 4.7 (0.026)
AZT	2 $\pm$ 1 ^a	1.2 $\pm$ 0.8 (0.23)	1.6 $\pm$ 0.2 (0.082)	0.7 $\pm$ 0.5 (0.00063)	n.d.	4.6 $\pm$ 1.6 (0.092)	n.d.	n.d.	n.d.
AraC	— ^b	— ^b	31.9 $\pm$ 1.7 (1.6)	9.8 $\pm$ 1.8 (0.0088)	14.8 $\pm$ 1.2 (0.086)	26.9 $\pm$ 7.4 (0.54)	280 $\pm$ 35.5 (0.112)	119 $\pm$ 19 (0.23)	38.5 $\pm$ 2.6 (0.042)
AraT	53.9 $\pm$ 5.4 ^a	53.7 $\pm$ 2.9 (10.2)	33.6 $\pm$ 2.5 (1.7)	5.5 $\pm$ 0.5 (0.005)	5.4 $\pm$ 1.7 (0.031)	68.2 $\pm$ 9.6 (1.36)	22.6 $\pm$ 5.2 (0.009)	n.d.	n.d.
AraA	16 $\pm$ 1 ^c	34.3 $\pm$ 1.9 (6.5)	24.7 $\pm$ 0.8 (1.3)	39.9 $\pm$ 3.5 (0.036)	11.9 $\pm$ 3.7 (0.069)	6.7 $\pm$ 1.3 (0.134)	1485 $\pm$ 77.4 (0.59)	1598 $\pm$ 148 (3)	1137 $\pm$ 52 (1.3)
F-AraA	43.4 $\pm$ 4.6	40.3 $\pm$ 1.7 (7.7)	31.3 $\pm$ 1.4 (1.6)	61.9 $\pm$ 2.8 (0.056)	24.6 $\pm$ 2.9 (0.14)	30 $\pm$ 4.9 (0.6)	2702 $\pm$ 38.7 (1.1)	1770 $\pm$ 158 (3.3)	1904 $\pm$ 126 (2.1)
ACV	n.d. ^a	n.d.	0.3 $\pm$ 0.1 (0.015)	0.2 $\pm$ 0.1 (0.00018)	n.d.	n.d.	n.d.	n.d.	n.d.
GCV	n.d.	n.d.	n.d.	0.7 $\pm$ 0.2 (0.00063)	2.9 $\pm$ 0.8 (0.017)	n.d.	n.d.	4.5 $\pm$ 1.6 (0.0086)	n.d.
3-dA	1.9 $\pm$ 0.6 ^a	0.6 $\pm$ 0.5 (0.11)	0.3 $\pm$ 0.03 (0.015)	0.8 $\pm$ 0.1 (0.00072)	1.8 $\pm$ 1 (0.01)	2.7 $\pm$ 0.5 (0.054)	77.2 $\pm$ 35.6 (0.031)	35.2 $\pm$ 3.3 (0.067)	n.d.
CdA	126 $\pm$ 4 ^a	114 $\pm$ 6.5 (21.7)	104 $\pm$ 3.5 (5.3)	220.7 $\pm$ 7.7 (0.2)	680 $\pm$ 164 (3.9)	61.6 $\pm$ 3 (1.2)	10144 $\pm$ 338 (4.1)	5531 $\pm$ 125 (10.5)	13183 $\pm$ 380 (14.5)
BVDU	54 $\pm$ 4 ^a	41.8 $\pm$ 2.5 (7.9)	42.9 $\pm$ 3.1 (2.2)	125 $\pm$ 3.1 (0.11)	640 $\pm$ 135 (3.7)	192.1 $\pm$ 6.4 (3.8)	1106 $\pm$ 48.3 (0.44)	1763 $\pm$ 59.7 (3.3)	1838 $\pm$ 95 (2.0)
D4T	n.d. ^c	0.14 $\pm$ 0.04 (0.027)	0.17 $\pm$ 0.15 (0.0087)	n.d.	n.d.	n.d.	n.d.	8 $\pm$ 2 (0.015)	n.d.
2-dR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.7 $\pm$ 7.1 (0.024)	n.d.

The activities were measured with the coupled spectrophotometric assay. The specific activity of each enzyme with Thd was set as 100%. Relative activities (in %) from 3–5 measurements $\pm$ SD are given. In parentheses, the specific activity of the mutants is compared with the specific activity of Dm-dNK with Thd as a substrate (100%).

^aData from Munch-Petersen *et al.* (2000).

^bThese substrates were characterized kinetically as the native substrates and can be found in Table I.

^cData from Knecht *et al.* (2000a).

n.d. = not detectable. Abbreviations: ACV, acyclic guanosine; (dd)Ado, (dideoxy)adenosine; AraA, 9- β -D-arabinofuranosyladenosine; AraC, 1- β -D-arabinofuranosylcytosine; AraT, 1- β -D-arabinofuranosylthymine; AZT, 3'-azido-2',3'-dideoxythymidine; BVDU, 5-bromo-vinyl-deoxyuridine; CdA, 2-chloro-deoxyadenosine; (dd)Cyd, (dideoxy)cytidine; D4T, 2',3'-didehydro-3'-deoxythymidine; 3-dA, 3'-deoxyadenosine; 2-dR, 2'-deoxyribose; Thd, thymidine; ddT, dideoxythymidine; dUrd, deoxyuridine; F-AraA, 2-fluoroadenine 9- β -D-arabinofuranoside; FdUrd, 5-fluorodeoxyuridine; GCV, ganciclovir; (dd)Guo, (dideoxy)guanosine.

BVDU, with a bromo-vinyl substitution at the 5-position of the Thd base, also did not exactly follow the expected pattern. Compared with the mutants Dm-dNK-V84A and Dm-dNK-V84S, the decrease in specific activity of the triple mutants was considerably less than for Thd (Table III). At present, we cannot explain the reasons for this unexpected behavior of these two analogs.

An improved catalytic efficiency for a nucleoside analog relative to the natural substrate can improve the

ability of deoxyribonucleoside kinases to mediate pro-drug-induced cell killing (Kokoris *et al.*, 1999; Knecht *et al.*, 2000a). Another example of this is Dm-dNK-V84A for which kinetic parameters indicate a more limited decrease in its efficiency for phosphorylation of ddC and AraC than for most of its natural substrates when compared with Dm-dNK. In the present work, some of our mutants (Table III) gained the ability to phosphorylate nucleoside analogs currently used in anti-viral therapy,

namely GCV, ACV and D4T, that were not converted by the wild-type Dm-dNK. This points towards interesting new enzyme–pro-drug combinations to be tested for suicide gene-mediated killing of cancer cells. HSV1-TK in combination with GCV can be regarded as the ‘workhorse’ for suicide gene-mediated cancer therapy by deoxyribonucleoside kinases (Loubière *et al.*, 1999). Despite the effectiveness of the HSV1-TK/GCV system, it remains important to try to improve it with the aim of maximizing therapeutic efficacy and therefore to search for new enzyme–pro-drug combinations. Wild-type Dm-dNK has been tested as a suicide gene in human cells with promising results for nucleoside analogs other than those used in combination with HSV1-TK, for example the nucleoside analogs gemcitabine and CdA (Zheng *et al.*, 2000). Because wild-type Dm-dNK has much higher turnover numbers for a number of substrates than HSV1-TK (Munch-Petersen *et al.*, 2000), some of the mutants might phosphorylate GCV or ACV more efficiently than HSV1-TK. Also some of the mutants might have gained an advantage over the wild-type Dm-dNK in phosphorylation of purine nucleoside analogs. However, like for Dm-dNK-V84A, which is superior to wild-type Dm-dNK in cell killing (Knecht *et al.*, 2000a), the efficiency (k_{cat}/K_m) of nucleoside analog phosphorylation in question needs to be determined and compared with the efficiency of phosphorylation of the natural substrates. Moreover, a superior ability to kill cells in a cellular test system needs to be shown.

Evolution of eukaryotic deoxyribonucleoside kinases

Basic principles in the evolution of enzymatic function have been reviewed recently (Gerlt and Babbitt, 2001). The present study represents a model case for reconstructing a possible evolutionary history for the eukaryotic enzyme family. Our results demonstrate that only 1–3 changes in one particular area of Dm-dNK can change its deoxyribonucleoside substrate specificities from Thd as the most preferred substrate to dCyd, dAdo or dGuo. This finding provides the basis to develop a model on the evolution of deoxyribonucleoside kinases.

In mammals, four deoxyribonucleoside kinases with specialized substrate specificities are found. Apparently, these four enzymes originated from a common progenitor multisubstrate kinase. The first gene duplication gave rise to the progenitor of TK1-like kinases and the progenitor of the TK2-, dCK/dGK-like kinases (Munch-Petersen *et al.*, 2000; Knecht *et al.*, 2002). Upon further rounds of duplication of the progenitor of TK2-, dCK/dGK-like kinases, the resulting copies each specialized for a limited number of substrates. Apparently, only a limited number of point mutations in each copy would have to accumulate to achieve this specialization. While the direct progenitor of Dm-dNK could be a specialized TK2-like thymidine-specific enzyme, apparently Dm-dNK has regained a broad substrate specificity. The reason for this retro-evolution could be that the *Drosophila* lineage at some point lost all other deoxyribonucleoside kinases, except the TK2-like enzyme. This enzyme was subsequently under evolutionary pressure to broaden its substrate specificity.

Materials and methods

Materials

³H-labeled deoxyribonucleosides were obtained from Amersham Pharmacia Biotech or Moravsek Biochemicals Inc., Brea, CA. Unlabeled nucleosides and nucleotides were from Sigma or ICN. Dideoxyadenosine (ddAdo), dideoxycytidine (ddCyd), dideoxyguanosine (ddGuo) and dideoxythymidine (ddThd) were a generous gift from Dr H.G. Ihlenfeldt, Roche Diagnostics. Oligonucleotides: MUT-for, 5'-CTAGTCTAGATAACGAGGGCAAAAAATGGCGGAGGCAGCATCCTGTGCCC; A110D-rev, 5'-GCAATAGCGATCGCTAAAAATGGAGCGCTCC; A110D-for, 5'-ATTTTATGCGATCGCTATTGCTTCGTGAG; M88R-rev, 5'-CGACTGCAGCCTGGTCAGCGTGACATAA-CTC; M88R-for, 5'-ACGCTGACCAGGCTGACGTGCGACACCGCC; V84M-rev, 5'-GGTCAGCGTCATATAACTCTGAAAGGGCATG; V84M-for, 5'-CAGAGTTATATGACGCTGACCATGCTG-CAG; V84S-rev, 5'-GGTCAGCGTGAATAACTCTGAAAGGGC-ATG; V84S-for, 5'-CAGAGTTATTCACGCTGACCATGCTG-CAG; pa-for, 5'-CGCGTG-GACATATGGCGGAGGCAGCATCC; pa-rev2, 5'-GATGAAGAATTC-TGTGGCGACCTCTGGCGCTT; linker1, 5'-TATGTTGGCCTCGT-TAT; linker2, 5'-CTAGATAACGAGGGCA-ACA; V84S-2, 5'-CAGAGTTATTCACGCTGACCATGCTG-CAG; V84A-3, 5'-CAGAGTTA-TGCCACGCTGACCAGGCTG-CAG; and V84A-4, 5'-GGTCAGCGT-GGCATAACTCTGAAAGGGCATG.

Site-directed mutagenesis and expression plasmids

For V84A, PCR on MuC (Knecht *et al.*, 2000a), harboring the V84A mutation, was performed with the primers pa-for and pa-rev2. The resulting PCR fragment was cut with *NdeI*–*EcoRI* and ligated into the vector pASK75-8His (Knecht *et al.*, 2002) cut by *XbaI*–*EcoRI*, with the help of a linker made by hybridization of the oligonucleotides linker1 and linker2. The resulting plasmid was named pASK-V84A.

For V84S, two overlapping PCR fragments were created with the primer combinations Mut-for/V84S-rev and pa-rev2/V84S-for. The vector pGEX-2T-rDmdNK (Munch-Petersen *et al.*, 2000) was used as a template. The two PCR fragments were isolated and used in another PCR together with the primers Mut-for/pa-rev2. The resulting fragment was cut with *XbaI* and *EcoRI* and subcloned into the *XbaI*–*EcoRI*-cut vector pASK75-8His. The resulting plasmid was named pASK-V84S. The construction of other point mutations was attempted in a similar way as described for V84S, but with the primers V84M-rev and V84M-for for V84M, with M88R-rev and M88R-for for M88R and with A110D-rev and A110D-for for A110D. When pASK-M88R was sequenced, it emerged that the plasmid lost its ribosome-binding site (RBS), making it impossible to express recombinant protein. Therefore, the RBS in pASK-M88R was restored by ligating an *XbaI*–*HincII* fragment from pASK-V84A and a *HincII*–*EcoRI* fragment from pASK-M88R into *XbaI*–*EcoRI*-cut vector pASK75-8His. When ‘pASK-A110D’ was sequenced, it was found to carry the double mutation M88R and A110D (which can only be explained by contamination of the second PCR round with primers M88R-rev and/or M88R-for, giving rise to a DNA shuffling reaction) and therefore was in fact pASK-M88R/A110D. Therefore, pASK-A110D was constructed as follows: an *XbaI*–*PstI* fragment from pASK-Dm-dNK (Knecht *et al.*, 2000b) and a *PstI*–*EcoRI* fragment from pASK-M88R/A110D were ligated into *XbaI*–*EcoRI*-cut vector pASK75-8His.

V84A/M88R/A110D and V84S/M88R/A110D were constructed in the following way: introduction of the third mutation was done in a two-step PCR procedure as described above for V84S. The plasmid pASK-M88R/A110D was used as a template for PCR, together with the mutagenic primers V84A-3 and V84A-4 for V84A/M88R/A110D and V84S-2 and V84S-rev for V84S/M88R/A110D.

Expression and purification of mutant Dm-dNKs

All mutants were expressed as fusion proteins with a C-terminal His₆ tag. The mutant proteins were expressed in *E. coli* BL21 for 4 h at 25°C after induction (at an OD_{600 nm} of 0.5–0.6) with 200 µg/l anhydrotetracycline in LB-ampicillin (100 µg/ml) medium. For purification of the recombinant proteins, *E. coli* cells were harvested at 4000 g for 20 min and resuspended in buffer A [20 mM sodium phosphate, 500 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 pH 7.4]. Cells were disrupted by sonification. After centrifugation for 30 min at 10 000 g, the supernatant was applied to a 1 ml HiTrap chelating column (Amersham Pharmacia Biotech) charged with Ni²⁺ ions. The column was washed with 10 vols of buffer A. The recombinant proteins were eluted with a linear gradient of 0–500 mM imidazole in buffer A. Recombinant Dm-dNK was

purified as described previously (Munch-Petersen *et al.*, 2000). Purity was determined by SDS–PAGE. SDS–PAGE was carried out according to the procedure of Laemmli (1970) and proteins were visualized by Coomassie Blue staining. The protein concentration was determined according to Bradford (1976) with bovine serum albumin as standard protein.

Enzyme assays

Nucleoside kinase activities were determined using tritium-labeled substrates. Alternatively, ADP production was measured by a spectrometric assay. Both assays were carried out as described by Munch-Petersen *et al.* (2000).

Analysis of kinetic data

Kinetic data were evaluated by non-linear regression analysis using the Michaelis–Menten equation $v = V_{\max} \times [S]/(K_m + [S])$ or the Hill equation $v = V_{\max} \times [S]^h/(K_{0.5}^h + [S]^h)$, as described by Knecht *et al.* (1996).

Acknowledgements

The skilful and committed technical assistance of Birgit Andersen and Marianne Lauridsen is gratefully acknowledged. This work was supported by grants from the Danish Research Council (STVF), the Danish Cancer Society, the Novo Nordisk research foundation, the John and Birthe Meyer Foundation and the Leo Foundation to J.P. and/or B.M.-P., and by grants from the Swedish Natural Science Research Council, the Swedish Strategic Research Foundation and the Swedish Cancer Foundation to H.E.

References

- Arnér, E.S.J. and Eriksson, S. (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol. Ther.*, **67**, 155–186.
- Bohman, C. and Eriksson, S. (1988) Deoxycytidine kinase from human leukemic spleen: preparation and characteristics of homogeneous enzyme. *Biochemistry*, **27**, 4258–4265.
- Bonini, C. *et al.* (1997) HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science*, **276**, 1719–1724.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**, 248–254.
- Christians, F.C., Scapozza, L., Cramer, A., Folkers, G. and Stemmer, W.P.C. (1999) Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling. *Nature Biotechnol.*, **17**, 259–264.
- Culver, K.W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H. and Blaes, R.M. (1992) *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science*, **256**, 1550–1552.
- Gerlt, J.A. and Babbitt, P.C. (2001) Divergent evolution of enzymatic function: mechanistically diverse superfamilies and functionally distinct suprafamilies. *Annu. Rev. Biochem.*, **70**, 209–246.
- Guettari, N., Loubiere, L., Brisson, E. and Klatzmann, D. (1997) Use of herpes simplex virus thymidine kinase to improve the antiviral activity of zidovudine. *Virology*, **235**, 398–405.
- Hatziz, P., Al-Madhoon, A., Jüllig, M., Petrakis, T.G., Eriksson, S. and Talianidis, I. (1998) The intracellular localization of deoxycytidine kinase. *J. Biol. Chem.*, **273**, 30239–30243.
- Johansson, K., Ramaswamy, S., Ljungcrantz, C., Knecht, W., Piškur, J., Munch-Petersen, B., Eriksson, S. and Eklund, H. (2001) Structural basis for substrate specificities of cellular deoxyribonucleoside kinases. *Nature Struct. Biol.*, **8**, 616–620.
- Jüllig, M. and Eriksson, S. (2000) Mitochondrial and submitochondrial localization of human deoxyguanosine kinase. *Eur. J. Biochem.*, **267**, 5466–5472.
- Knecht, W., Bergjohann, U., Gonski, S., Kirschbaum, B. and Löffler, M. (1996) Functional expression of a fragment of human dihydroorotate dehydrogenase by means of the baculovirus expression vector system and kinetic investigation of the purified recombinant enzyme. *Eur. J. Biochem.*, **240**, 292–301.
- Knecht, W., Munch-Petersen, B. and Piškur, J. (2000a) Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*. *J. Mol. Biol.*, **301**, 827–837.

- Knecht, W., Munch-Petersen, B. and Piškur, J. (2000b) Polyclonal antibodies against the ultrafast multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*. *Adv. Exp. Med. Biol.*, **486**, 263–266.
- Knecht, W., Petersen, G.E., Munch-Petersen, B. and Piškur, J. (2002) Deoxyribonucleoside kinases belonging to the thymidine kinase 2 (TK2)-like group vary significantly in substrate specificity, kinetics and feed-back regulation. *J. Mol. Biol.*, **315**, 529–540.
- Kokoris, M.S., Sabo, P., Adman, E.T. and Black, M.E. (1999) Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant. *Gene Ther.*, **6**, 1415–1426.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lal, S., Lauer, U.M., Niethammer, D., Beck, J.F. and Schlegel, P.G. (2000) Suicide genes: past, present and future perspectives. *Immunol. Today*, **21**, 48–54.
- Loubiere, L., Tiraby, M., Cazaux, C., Brisson, E., Grisoni, M., Zhao-Emonet, J.C., Tiraby, G. and Klatzmann, D. (1999) The equine herpes virus 4 thymidine kinase is a better suicide gene than the human herpes virus 1 thymidine kinase. *Gene Ther.*, **6**, 1638–1642.
- Mandel, I. *et al.* (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nature Genet.*, **29**, 337–341.
- Munch-Petersen, B., Cloos, L., Tyrsted, G. and Eriksson, S. (1991) Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J. Biol. Chem.*, **266**, 9032–9038.
- Munch-Petersen, B., Piškur, J. and Søndergaard, L. (1998) Four deoxynucleoside kinase activities from *Drosophila melanogaster* are contained within a single monomeric enzyme, a new multifunctional deoxynucleoside kinase. *J. Biol. Chem.*, **273**, 3926–3931.
- Munch-Petersen, B., Knecht, W., Lenz, C., Søndergaard, L. and Piškur, J. (2000) Functional expression of a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* and its C-terminal deletion mutants. *J. Biol. Chem.*, **275**, 6673–6679.
- Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S. and Elpeleg, O. (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nature Genet.*, **29**, 342–344.
- Wang, L. and Eriksson, S. (2000) Cloning and characterization of full-length mouse thymidine kinase 2: the N-terminal sequence directs import of the precursor protein into mitochondria. *Biochem. J.*, **351**, 469–476.
- Wang, L., Karlsson, A., Arnér, E. and Eriksson, S. (1993) Substrate specificity of mitochondrial 2'-deoxyguanosine kinase. Efficient phosphorylation of 2-chlorodeoxyadenosine. *J. Biol. Chem.*, **268**, 22847–22852.
- Wild, K., Böhner, T., Folkers, G. and Schulz, G.E. (1997) The structures of thymidine kinase from herpes simplex virus type 1 in complex with substrates and a substrate analogue. *Protein Sci.*, **6**, 2097–2106.
- Zheng, X., Johansson, M. and Karlsson, A. (2000) Retroviral transduction of cancer cell lines with the gene encoding *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. *J. Biol. Chem.*, **275**, 39125–39129.

Received December 13, 2001; revised and accepted February 4, 2002