0026-895X/98/020270-04$3.00/0

MOLECULAR PHARMACOLOGY, **53:**270–273 (1998).

Substrate Specificity of Human Recombinant Mitochondrial Deoxyguanosine Kinase with Cytostatic and Antiviral Purine and Pyrimidine Analogs

ANITA HERRSTRÖM SJÖBERG, LIYA WANG, and STAFFAN ERIKSSON

Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Biomedical Center, Uppsala, Sweden

Received May 30, 1997; Accepted October 27, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

Deoxyguanosine kinase (dGK) is an enzyme responsible for the phosphorylation of purine deoxynucleosides in mitochondria of mammalian cells. Its role in activation of pharmacologically used nucleoside analogs is not well understood, because of the low levels of dGK found in tissue extracts and its inactivation during purification. The cDNA for dGK was recently cloned and expressed in *Escherichia coli*. Here we present an improved procedure for expression and purification of a highly active form of human recombinant dGK. The enzyme showed a broad substrate specificity toward natural purine and pyrimidine deoxynucleosides as well as toward important nucleoside analogs. The K_m and V_{max} values for deoxyguanosine, deoxyinosine, deoxyadenosine, and deoxycytidine were 4, 13, 460, 330 μM and 43, 330, 430 and 60 nmol/min/mg of protein, respectively. Antileukemic purine analogs such as arabinosyl guanine, 2-chloro-2′-deoxyadenosine, 2-chloro-2′-*arabino*-fluoro-2′-deoxyadenosine, and 2-fluoro-arabinosyl-adenine were phosphorylated as efficiently by dGK as the natural nucleoside substrates. This is the first report in which 2-fluoro-arabinosyl-adenine and 2-chloro-2′-*arabino*-fluoro-2′-deoxyadenosine were shown to be good substrates for dGK. The antiviral analogs dideoxyinosine and arabinosyl adenine also showed significant activity with dGK, as did several pyrimidine analogs (e.g., the cytostatic drugs 5-fluoro-2′-deoxycytidine and difluorodeoxycytidine). The broad specificity of dGK described here may change our understanding of the mechanisms responsible for the efficacy and mitochondrial toxicity of several nucleoside analogs.

Mammalian deoxyguanosine kinase (dGK; nucleoside triphosphate: deoxyguanosine 5′-phosphotransferase, EC 2.7.1.113) is known to catalyze the phosphorylation of purine deoxynucleosides and their analogs, using a nucleoside triphosphate as phosphate donor (Gower *et al.*, 1979; Yamada *et al.*, 1982; Sarup and Fridland, 1987; Park and Ives, 1988; Arnér and Eriksson, 1995; Wang *et al.*, 1993, 1996; Johansson and Karlsson, 1996). The cDNA for dGK was recently cloned and shown to code for a 32-kDa protein with an amino-terminal leader sequence characteristic of mitochondrial proteins (Wang *et al.*, 1996; Johansson and Karlsson, 1996). This result confirms earlier biochemical studies of the subcellular localization of dGK and agrees with the ubiquitous distribution of the enzyme in all tissues at approximately equal levels (Arnér and Eriksson, 1995). The active form of the recombinant as well as the natural dGK is a dimer of two 29-kDa subunits and dGuo, dAdo, dIno, and several pharmacologically important analogs [e.g., arabinosyl guanine (AraG) and 2-chloro-deoxyadenosine (CdA)] were shown to be good substrates for the enzyme (Wang *et al.*, 1993; Arnér and Eriksson, 1995).

The coding sequence for dGK showed 46% homology with the coding sequences of cytosolic deoxycytidine kinase (Chottiner *et al.*, 1991; Johansson and Karlsson, 1996; Wang *et al.*, 1996). Thus, dGK and dCK belong to the same enzyme family, which also includes the Herpes virus thymidine kinase family (Harrison *et al.*, 1991; Gentry, 1992; Eriksson and Wang, 1997). In light of this genetic relationship, it is not surprising that dCK and dGK have overlapping substrate specificities (Chottiner *et al.*, 1991; Arnér and Eriksson, 1995; Wang *et al.*, 1996).

A major problem in the biochemical characterization of both natural and recombinant dGK has been that the enzyme is unstable and has a high tendency to aggregate during purification and storage. Therefore, the specific activity of most preparations of pure dGK tested so far has been low compared with that of the other deoxynucleoside kinases,

This work was supported by Grant B95–03X-06230–14C from the Swedish Medical Research Council and Grant BM44-CT96–0476 from the European community.

ABBREVIATIONS: dGK, deoxyguanosine kinase; dCK, deoxycytidine kinase; SDS, sodium dodecyl sulfate; AraA, arabinosyl adenine; AraG, arabinosyl guanine; 2-F-AraA, 2-fluoro-arabinosyl-adenine; CdA, 2-chloro-2′-deoxyadenosine; CAFdA, 2-chloro-2′-*arabino*-fluoro-2′-deoxyadenosine; dFdC, 2′-difluoro-2′-deoxycytidine; ddA, dideoxyadenosine; ddC, dideoxycytidine; ddI, dideoxyinosine; 5-FdC, 5-fluoro-2′-deoxycytidine; dAdo, deoxyadenosine; dCyd, deoxycytidine; dGuo, deoxyguanosine; dIno, deoxyinosine.

suggesting that earlier determinations of the substrate specificity of dGK have been done with only partially active enzyme.

In the present investigation, conditions to express and stabilize recombinant dGK were optimized and the final specific activity of pure dGK was at least 10-fold higher than that reported earlier (Wang *et al.*, 1993, 1996). The capacity of this highly active dGK to phosphorylate several deoxynucleoside analogs was determined and shown to include a broad spectrum of compounds. These results may lead to a reinterpretation of the mechanism of activation of several clinically important nucleoside analogs.

Experimental Procedures

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) and [5-^{3}H]Deoxycytidine (24 Ci/mmol) were obtained from Amersham (Little Chalfont England). [8-^{3}H]Guanine-8-D-arabinofuranoside (6.5 Ci/mmol), [8-^{3}H]Chlorodeoxyadenosine (4 Ci/mmol), and [8-^{3}H]Deoxyguanosine (5.8 Ci/mmol) were purchased from Moravek (Brea, CA).

Methods

Expression and purification of recombinant dGK protein. One colony of pLys S BL21(DE3) containing the dGK coding sequence in a pET-9d vector (Wang *et al.*, 1996) was expanded to a 1-liter culture medium (10 g of tryptone and 5 g of NaCl/liter, 50 μg/ml chloramphenicol, 1× M9 salt, 10 mM $MgSO_4$, 0.4% glucose) containing 50 μg/ml kanamycin and grown at 37° until $A_{600nm} \approx 0.700$. The culture was then moved to 25° and induced with 0.2 mM isopropyl-β-thiogalactopyranoside for 16 hr. Bacteria were pelleted by centrifugation at 2,000 × *g* for 30 min, and lysed in 5 volumes of lysis buffer (50 mM Tris·HCl, pH 7.6, 0.5 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA) by freezing and thawing three times. The lysate was centrifuged at 100,000 × *g* for 90 min and the supernatant saved. Recombinant dGK was purified by metal affinity chromatography (TALONE; Clontech Palo Alto, CA) as described by the supplier. dGK was eluted with 250 mM imidazole in the presence of 0.1 M NaCl, 0.1% Triton X 100, and 0.1 mM ATP. Fractions were analyzed with SDS polyacrylamide gel electrophoresis and the activity determined with several deoxynucleosides.

Enzyme assays. dGK activity was determined using [^{3}H]AraG (or the other radioactively labeled nucleosides) as substrate as described previously (Wang *et al.*, 1993, 1996). The reaction buffer contained 50 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 15 mM NaF, 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.1% Triton X-100, and dGK in a total volume of 50 μl. The phosphoryl transfer assay was performed with 50 mM Tris·HCl pH 7.6, 5 mM $MgCl_2$, 100 mM KCl, 15 mM NaF, 10 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 0.05 μM [γ-^{32}P]ATP (10 mCi/ml), 100 μM ATP, 100 ng of dGK, and usually 10 μM or 100 μM of nucleoside in a total volume of 50 μl. The phosphorylated products were separated by thin layer chromatography and quantified as described (Wang *et al.*, 1993).

Removal of the histidine-tag sequence. Thrombin cleavage of purified recombinant dGK was done with Thrombin (Novagen, Madison, WI) with a ratio of 10 units to 1 mg of protein in the presence of 10 mM β-mercaptoethanol. Incubation was done at 22° for 48 hr. Aliquots of the cleaved protein were analyzed by 14% SDS gel electrophoresis.

Results and Discussion

Expression and Purification of a Highly Active Form of dGK

An efficient expression system for recombinant dGK, using the pET-9d vector transfected into the BL21(DE3) bacteria, has been described previously (Wang *et al.*, 1996). A histidine-tag sequence as well as a Thrombin cleavage site is fused to the amino terminus of the coding sequence of human dGK. The start sequence of dGK corresponds to the peptide sequence MAKSPLE- (Wang *et al.*, 1996); this may be a truncated version of human dGK, because the cDNA of a dGK leader peptide 17 amino acids longer has been described previously (Johansson and Karlsson, 1996). Nevertheless, the activity and the specificity of the shorter version of dGK are very similar to that of the longer form (Johansson and Karlsson, 1996; Wang *et al.*, 1996); inside the mitochondria, the amino-terminal leader peptide is removed (Gavel and von Heijne, 1990; Nakai and Kanehisa, 1992). The specific activity of the earlier purified recombinant dGK was dGMP formed at the rate of 5 nmol/min/mg of protein (Wang *et al.*, 1996), which is similar to that observed with purified bovine brain dGK (Wang *et al.*, 1993). This specific activity is almost 10–50-fold lower than that of other deoxynucleoside kinases belonging to the same family [e.g., deoxycytidine kinase and thymidine kinase 2 (Arnér and Eriksson, 1995; Eriksson and Wang, 1997)].

In an attempt to optimize the expression and stability of recombinant dGK, we analyzed whole bacterial cells by SDS polyacrylamid gel electrophoresis and found, after induction, a very prominent dGK protein band. The soluble extracts from the same cells showed a much lower level of dGK, which indicates that a large part of dGK was insoluble. We could improve the yield of soluble enzyme by reducing the temperature during induction to 25°, decreasing the concentration of the inducer isopropyl-β-thiogalactopyranoside from 1 to 0.2 mM and increasing the induction period from 2 hr to 16 hr. These modifications gave a significantly higher yield of soluble dGK.

Addition of 5 mM mercaptoethanol as a reducing agent stabilized the enzyme during metal affinity chromatography. The presence of the nonionic detergent Triton X-100 was also essential as observed earlier with bovine liver dGK (Park and Ives, 1988). Addition of 0.1 mM ATP during the affinity chromatography step further improved the yields. The specific activity of the final preparation of dGK, which was more than 98% pure (Fig. 1), was dGMP formed at the rate of 40–50 nmol/min/mg. The removal of the histidine-tag sequence could be done by incubation with Thrombin for 48 hr at 22° (Fig. 1) without any significant loss of enzyme activity. Thus, we conclude that with the improved expression and purification procedure, a pure dGK could be prepared, with or without the histidine-tag sequence, that would be at least 10 times more active than earlier preparations. There was no significant difference in the kinetic parameters of dGK with or without the histidine tag as described below.

Substrate Specificity and Kinetic Parameters of Highly Active dGK

The capacity of recombinant dGK prepared by the improved procedure to phosphorylate purine and pyrimidines was tested with several nucleosides, using ATP as phosphate donor. The K_m value for dGK with dGuo was 4 μM, which is close to that reported earlier, but the V_{max} was 10-fold higher (Wang *et al.*, 1993, 1996) (Table 1). The two other natural purine deoxynucleosides, dAdo and dIno (Table 1), were also substrates and, in the case of dAdo, exhibited a much higher K_m value and thus a much-reduced efficiency. However, with dIno, the K_m was somewhat higher than with dGuo, but the V_{max} was 8-fold higher and the efficiency was severalfold higher than with any other substrate (Table 1). This result implies that dIno may be the most important substrate for dGK *in vivo*.

The capacity of dGK to use dCyd as substrate has been reported (Wang *et al.*, 1996) and may be explained by the close similarities in primary structure of the two enzymes (Johansson and Karlsson,

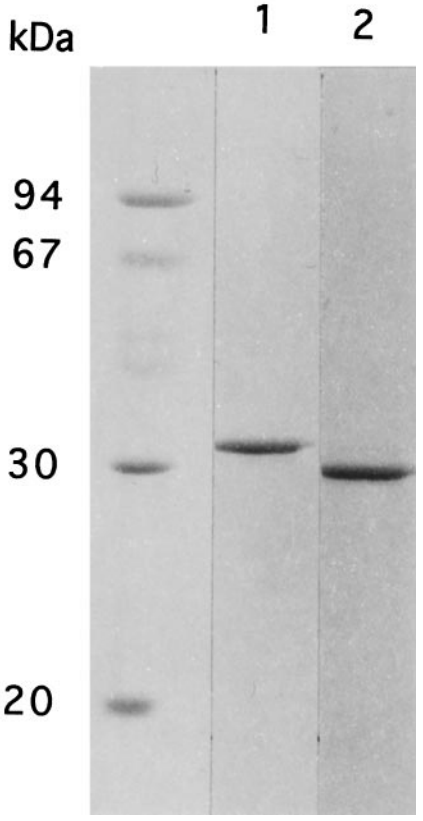

Fig. 1. SDS gel electrophoresis of dGK after metal affinity chromatography (*lane 1*) and after treatment with Thrombin to remove the histidine-tag sequence (*lane 2*).

TABLE 1

Kinetic constants of recombinant dGK with nucleoside substrates

The values are those determined with radioactive nucleosides and 5 mM ATP as phosphate donor. Values in parentheses are those determined with the phosphoryl transfer assay and 100 μM [γ-^{32}P]ATP as phosphate donor. The values are from one experiment repeated at least twice with similar results.

Substrate	K_m		V_{max}		Efficiency (V_{max}/K_m)	
		μM	nmol/mg/min			
dGuo	4	(4)	43	(48)	10.8	(12)
AraG	33		204		6	
dCyd	336		59		0.2	
dAdo	467		429		0.9	
CAFdA	56		380		6.7	
CdA	78		770		9.9	
2-F-AraA		(460)		(380)		(0.8)
dIno		(12)		(390)		(32.5)

1996; Wang *et al.*, 1996). We determined that the K_m and V_{max} for dCyd with the active form of dGK and the efficiency was about 2% compared with dGuo (Table 1). Although the activity of dGK with pyrimidines is low, it may still be of physiological significance, as discussed below.

Several important antitumor purine nucleoside analogs (e.g., AraG, CdA, CAFdA and 2-F-AraA) all require activation by kinases to be toxic to the target cells. These analogs are all promising antileukemic drugs in clinical use or in the advanced clinical testing stages (Carson *et al.*, 1984, 1992; Plunkett and Saunders, 1991; Kurtzberg, 1993) and they are all considered to be phosphorylated primarily by dCK. With the active recombinant dGK, the kinetic parameters for these purine analogs were determined; the K_m value for AraG was higher than for dGuo, as was the V_{max} value (Table 1). The two deoxyadenosine analogs were both better substrates for dGK than dAdo and, in case of CdA, the efficiency was as high as with dGuo. The high activity with dGK may be a contributing factor for the cytotoxic activity of CdA and CAFdA in certain tumors and tissues.

2-F-AraA was also a relatively good substrate for dGK; the kinetic parameters for this analog and dIno were determined with the phosphoryl transfer assay, in which the ATP concentration is 0.1 mM instead of 5 mM as in the standard assay (Table 1). Only minor differences in the K_m and V_{max} values for dGuo, AraG, and dAdo were observed with the two different assay systems (Table 1). The removal of the histidine-tag sequence from dGK (Fig. 1) led to an enzyme preparation with very similar kinetic properties. The K_m and V_{max} values for dGuo, AraG, and dAdo demonstrated a maximal difference of ± 10% with the two forms of dGK (data not shown). Because the histidine tag seemed to have no direct effect on the substrate kinetics of dGK, we routinely used this form of the enzyme.

A series of other antitumor and antiviral deoxynucleoside analogs were tested with dGK using the phosphoryl transfer assay and 10 and 100 μM substrate concentrations (Table 2). ddC, ddI, and ddA are analogs that show good anti-HIV activity *in vitro*; ddC and ddI are registered for use in anti-HIV treatment (Johns, 1997). The activation of ddC is known to be carried out by dCK, and ddI and ddA are phosphorylated mainly via the phosphotransfer activity of cytosolic 5′-nucleotidase (Arnér and Eriksson, 1995; Johns, 1997). We observe here that ddI can be phosphorylated by dGK, albeit at a low level (Table 2). ddA and ddC showed no detectable activity with dGK in this assay (Table 2). No activity was observed with the anti-Herpes virus analogs acyclovir and ganciclovir (Table 2) in accordance with earlier results (Wang *et al.*, 1993; Arnér and Eriksson, 1995). AraA is active against Herpes viruses (Gentry, 1992); earlier studies have failed to show activity with dGK (Gower *et al.*, 1979; Sarup and Fridland, 1987; Johansson and Karlsson, 1996). However, we observed here a low but significant level of phosphorylation of AraA by dGK (Table 2).

Several deoxycytidine analogs (e.g., arabinosyl cytosine and 5-FdC) are efficient antileukemic agents, and dFdC shows very promising activity against solid tumors as well (Chabner, 1996). We observe here for the first time that dGK has the capacity to phosphorylate 5-FdC and dFdC with ~ 2–10% of the activity with dGuo.

It is still unclear whether the activity of dGK contributes significantly to the toxicity of natural or pharmacologically active cytostatic and antiviral analogs *in vivo* However, there are strong indications that this is the case; for example, Snyder *et al.* (1994) have shown that in mutant mice that are deficient in purine nucleoside phos-

TABLE 2

Substrate specificity of dGK with nucleosides using the phosphoryl transfer assay

The activity was determined as described with 100 μM [γ-^{32}P]ATP as phosphate donor. The values show the activity relative to that with 10 μM dGuo, which was set to 1.0. The values are from one experiment repeated at least twice with very similar results.

Substrate	Activity		Source[a]
	10 μM	100 μM	
dGuo	1.0	1.2	S
AraG	1.2	1.7	W
2′-Difluoro-2′-deoxyguanosine	1.7	1.8	E
Gangciclovir	<0.01	0.05	M
Acyclovir	<0.01	<0.01	M
ddA	<0.01	<0.01	S
AraA	0.03	0.1	S
2-F-AraA	0.16	0.8	B
dIno	1.2	2.5	S
ddI	0.06	2.5	S
5-FdC	0.06	0.8	M
Arabinosyl cytosine	<0.01	<0.01	S
ddC	<0.01	<0.01	S
dFdC	0.01	0.06	E

[a] The nucleosides were obtained from S, Sigma Chemical, St. Louis, MO; B, Berlex, Alameda, CA; or as gifts from E, Eli Lilly, Indianapolis, IN; W, Burroughs Wellcome, Research Triangle Park, NC; M, Medivir AB, Huddinge, Sweden.

phorylase activity, leading to high plasma concentration of dGuo, there was a compensatory decrease in dGK levels to prevent accumulation of toxic levels of deoxyguanosine nucleotides. This result indicates that phosphorylation by dGK of natural purine nucleosides was detrimental in situations with altered nucleoside catabolism. The cytotoxicity occurs most likely through inhibition of nuclear DNA synthesis, although effects on the mitochondrial DNA synthesis were not determined in this case.

Recent experiments with dCK-deficient human CEM cells showed that these cells were still sensitive to the cytotoxic effects of 2-F-AraA. The activating enzyme in this case is probably dGK (Albertioni *et al.*, 1997; Lotfi *et al.*, 1997). The results suggest that dGK activity is important for the formation of toxic 2-F-AraA nucleotide levels in cells; it is still not known whether inhibition of mitochondrial DNA synthesis is a contributing factor in cell killing by this drug. The fact that dGK has a much broader substrate specificity, including both purine and pyrimidine nucleosides, may lead to a reinterpretation of the mechanism of toxicity for several antiviral and cytostatic drugs.

References

Albertioni F, Spasokoutkotskaja T, Peterson B, Knaust E, Eriksson S, and Liliemark J (1997) Mechanism of 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA) resistance in a human lymphoid, CCRF-CEM cell line (abstract), in Proceedings of the American Association for Cancer Research, vol. 38; 1997 Apr 12–16; San Diego, CA. p. 103, American Association for Cancer Research, Philadelphia.

Arnér ESJ and Eriksson S (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol Ther* **67**:155–186.

Carson D, Wasson D, and Beutler E (1984) Antileukemic and immunosuppressive activity of 2-chloro-2′-deoxyadenosine. *Proc Natl Acad Sci USA* **81**:2232–2236.

Carson D, Wasson D, Esparza L, Carrera C, Kipps T, and Cottam H (1992) Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine. *Proc Natl Acad Sci USA* **89**:2970–2974.

Chabner BA (1996) Cytidine analogues, in *Cancer Chemotherapy and Biotherapy* (Chabner BA and Longo DL, eds) pp 213–233, Lippincott-Raven, Philadelphia.

Chottiner EG, Shewach DS, Datta NS, Ashcraft E, Gribbin D, Ginsburg D, Fox IH, and Mitchell BS (1991) Cloning and expression of human deoxycytidine kinase cDNA. *Proc Natl Acad Sci USA* **88**:1531–1535.

Eriksson S and Wang L (1997) Substrate specificities, expression, and primary sequences of deoxynucleoside kinases; implications for chemotherapy. *Nucleosides Nucleotides* **16**:653–659.

Gavel Y and von Heijne G (1990) Cleavage-site motifs in mitochondrial targeting peptides. *Protein Eng* **4**:33–37.

Gentry GA (1992) Viral thymidine kinases and their relatives. *Pharmacol Ther* **54**:319–355.

Gower WJ, Carr MC, and Ives DH (1979) Deoxyguanosine kinase. Distinct molecular forms in mitochondria and cytosol. *J Biol Chem* **154**:2180–2183.

Harrison PT, Thompson R, and Davison AJ (1991) Evolution of herpesvirus thymidine kinases from cellular deoxycytidine kinase. *J Gen Virol* **72**:2583–2586.

Johansson M and Karlsson A (1996) Cloning and expression of human deoxyguanosine kinase cDNA. *Proc Natl Acad Sci USA* **93**:7258–7262.

Johns DG (1997) Pharmacology of antiretroviral nucleoside analogs, in *Anti-HIV nucleosides: Past, present and future*. (Mitsuya H, ed) pp 23–50, R. G. Landes, Austin, TX.[T]

Kurtzberg J (1993) Guanine arabinoside as a bone marrow-purging agent. *Ann N Y Acad Sci* **685**:225–36.

Lotfi K, Liliemark J, Peterson B, Spasokoutkotskaja T, Eriksson S, and Albertioni F (1997) Cross-resistance between purine and pyrimidine analogs in human lymphoid cell lines, CCRF-CEM cells selected to resistance to cladribine and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (abstract). *Proc Am Assoc Cancer Res* **38:**102.

Nakai K and Kanehisa M (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14**:897–911.

Park I and Ives DH (1988) Properties of a highly purified mitochondrial deoxyguanosine kinase. *Arch Biochem Biophys* **266**:51–60.

Plunkett W and Saunders P (1991) Metabolism and action of purine nucleoside analogs. *Pharmacol Ther* **49**:239–268.

Sarup JC and Fridland A (1987) Identification of purine deoxyribonucleoside kinases from human leukemia cells: substrate activation by purine and pyrimidine deoxyribonucleosides. *Biochemistry* **26**:590–597.

Snyder FF, Jenuth JP, Dilay JE, Fung E, Lightfoot T, and Mably ER (1994) Secondary loss of deoxyguanosine kinase activity in purine nucleoside phosphorylase deficient mice. *Biochim Biophys Acta* **1227**:33–40.

Wang L, Hellman U, and Eriksson S (1996) Cloning and expression of human mitochondrial deoxyguanosine kinase cDNA. *FEBS Lett* **390**:39–43.

Wang L, Karlsson A, Arnér ESJ, and Eriksson S (1993) Substrate specificity of mitochondrial 2′-deoxyguanosine kinase efficient phosphorylation of 2-chlorodeoxyadenosine. *J Biol Chem* **268**:22847–22852.

Yamada Y, Goto H, and Ogasawara N (1982) Deoxyguanosine kinase from human placenta. *Biochim Biophys Acta* **709**:265–272.

Send reprint requests to: Dr. Staffan Eriksson, Dept. of Veterinary Medical Chemistry, Biomedical Centre, P.O. Box 575, S 75123 Uppsala, Sweden. E-mail: staffan.eriksson@vmk.slu.se