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

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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, deoxycytidine, deoxyadenosine, and deoxycytidine were 4, 13, 460, 330 $\mu M$ 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'-'arabinofluoro-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'-deoxyctydine 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.

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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'-difuoro-2'-deoxycytidine; dDA, deoxyadenosine; dDC, deoxycytidine; ddl, dideoxyinosine; 5-FdC, 5-fluoro-2'-deoxycytidine; dAdo, deoxyadenosine; dCyd, deoxycytidine; dGuo, deoxyguanosine; dIno, deoxyinosine.
suggested 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**

$[^{32}P]ATP$ (3000 Ci/mmole) and $[^{3}H]Deoxyctydine$ (24 Ci/mmole) were obtained from Amersham (Little Chalfont England). $[^{3}H]Guanosine-8'-O-arabinofuranoside$ (6.5 Ci/mmole), $[^{3}H]Chlorodeoxyadenosine$ (4 Ci/mmole), and $[^{3}H]Deoxyguanosine$ (5.8 Ci/mmole) 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 the 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 $\mu$g/ml chloramphenicol, 1$\times$ M9 salt, 10 mM MgSO$_4$, 0.4% glucose) containing 50 $\mu$g/ml kanamycin and grown at 37$^\circ$ until A$_{600nm}$ = 0.7. The culture was then moved to 25$^\circ$ and induced with 0.2 mM isopropyl-$\beta$-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 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 5 mM $\beta$-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. The presence of the nonionic detergent Triton X-100 was also essential as observed earlier with bovine liver 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 (Arner 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 polyacrylamide 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$^\circ$, decreasing the concentration of the inducer isopropyl-$\beta$-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$^\circ$ (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 $\mu$M, 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 several-fold 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, 1993). The expression and purification of a highly active form of dGK

**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 mmol/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 (Arner and Eriksson, 1995; Eriksson and Wang, 1997)).
Several important antitumor purine nucleoside analogs (e.g., AraG, CdA, CAFdA and 2-F-AraA) all require activation by kinases discussed below.

The values are those determined with radioactive nucleosides and 5 mM 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.

### TABLE 1

Kinetic constants of recombinant dGK with nucleoside substrates

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

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 pyrimidine analogs (e.g., AraG, CAFdA and 2-F-AraA) all require activation by kinases to be toxic to the target cells. These analogs are all promising antitumor drugs in clinical use or in the advanced clinical testing stages. 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 10% 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 phosphorl 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).

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 deoxycytidine analogs were tested with dGK using the phosphorl 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 (Arné 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 antiviral virus analogs acyclovir and ganciclovir (Table 2) in accordance with earlier results (Wang et al., 1993; Arné and Eriksson, 1995). AraA is active against Herpes viruses (Gentry, 1992); earlier studies have failed to show activity with dGK (Gower et al., 1979; Surup 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 phosphotransferase, 5-FdC has a reduced effect on tumor growth.

### TABLE 2

Substrate specificity of dGK with nucleosides using the phosphoryl transfer assay

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity ($10 \mu M$)</th>
<th>Activity ($100 \mu M$)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGuo</td>
<td>1.0</td>
<td>1.2</td>
<td>S</td>
</tr>
<tr>
<td>AraG</td>
<td>1.2</td>
<td>1.7</td>
<td>W</td>
</tr>
<tr>
<td>2'-Difluoro-2'-deoxyguanosine</td>
<td>1.7</td>
<td>1.8</td>
<td>E</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>M</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>M</td>
</tr>
<tr>
<td>ddA</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>S</td>
</tr>
<tr>
<td>AraA</td>
<td>0.03</td>
<td>0.1</td>
<td>S</td>
</tr>
<tr>
<td>2-F-AraA</td>
<td>0.16</td>
<td>0.8</td>
<td>B</td>
</tr>
<tr>
<td>dIno</td>
<td>1.2</td>
<td>2.5</td>
<td>S</td>
</tr>
<tr>
<td>ddI</td>
<td>0.06</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>5-FdC</td>
<td>0.06</td>
<td>0.8</td>
<td>M</td>
</tr>
<tr>
<td>Arabinosyl cytosine</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>S</td>
</tr>
<tr>
<td>ddC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>S</td>
</tr>
<tr>
<td>dFdC</td>
<td>0.01</td>
<td>0.06</td>
<td>E</td>
</tr>
</tbody>
</table>

*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.
phosphorylase 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 pyrimidine 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


