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**SHORT COMMUNICATION**

# Phosphorylation of the Anti-HIV Compound (*S,S*)-Isodideoxyadenosine by Human Recombinant Deoxycytidine Kinase

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**ABSTRACT.** (*S,S*)-Isodideoxyadenosine [(*S,S*)-isoddA] is an anti-HIV active compound discovered in our laboratory. However, its cellular mechanism of action, particularly the critical first stage of phosphorylation, is not understood. IsoddA is not phosphorylated by adenosine kinase. Also, because it is not a substrate for adenosine deaminase, it would not be activated by the pathway taken by ddA, i.e. via 5′-nucleotidase phosphorylation of ddI and conversion of ddIMP to ddAMP. However, we have discovered that human recombinant 2′-deoxycytidine kinase (dCK) phosphorylates (*S,S*)-isoddA. The enzyme kinetic data revealed that the extent of monophosphorylation of this L-related nucleoside was comparable to that found with ddA. (*S,S*)-IsoddATP is among the most potent inhibitors of HIV reverse transcriptase known, which suggests that the observed low efficiency of phosphorylation of this compound by dCK is a key factor that limits the capacity of human lymphocytes to make (*S,S*)-isoddA an exceptionally active anti-HIV agent. BIOCHEM PHARMACOL **60**;10:1505–1508, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** (*S,S*)-isodideoxyadenosine; anti-HIV activity; deoxycytidine kinase; adenosine kinase; monophosphorylation

4(*S*)-(6-Amino-9*H*-purin-9-yl)tetrahydro-2(*S*)-furanmethanol or (*S,S*)-isodideoxyadenosine [(*S,S*)-isoddA] (Fig. 1) is a conceptually novel dideoxynucleoside discovered in our laboratory, in which the glycosyl bond has been transposed from the normal anomeric position to the 2′-position [1]. It is an isomeric analog of β-D-ddA, but is very stable with respect to "glycosyl" bond cleavage under acidic conditions. IsoddA is resistant to deamination by mammalian adenosine deaminase [2]. It inhibits the replication of wild-type HIV-1 and HIV-2 and exhibits synergistic inhibition of HIV in combination with AZT,† ddI, and FTC [2]. (*S,S*)-IsoddA is converted in cells to its triphosphate [(*S,S*)-isoddATP], which can be identified by HPLC. (*S,S*)-IsoddATP is one of the most potent known inhibitors of HIV reverse transcriptase, and its $K_i$ (16 nM) with activated calf thymus DNA as the nucleic acid substrate [2] is comparable to data obtained for AZTTP [3].

The mechanism of the conversion of (*S,S*)-isoddA to (*S,S*)-isoddATP has not been investigated. In particular, the enzyme responsible for the critical first stage of phosphorylation is not known. It is well established that dCK (EC 2.7.1.74) plays a major role in the initial phosphorylation of several nucleoside analogs of therapeutic interest and that the anti-HIV activity of these compounds is dependent on this phosphorylation [4–9]. For example, dCK is known to phosphorylate the anti-HIV compounds ddC and d4T [5, 8]. Mammalian cell lines genetically deficient in dCK are resistant to the cytotoxic and antiviral effects of ddC, which is apparently not phosphorylated to the nucleoside monophosphate level in these cells [10]. In the case of ddA, some direct phosphorylation to ddAMP by adenosine kinase or dCK may take place. However, because of rapid deamination to ddI, the major pathway for the conversion of ddA to ddAMP is through phosphorylation by 5′-nucleotidase to ddIMP and subsequent conversion of ddIMP to ddAMP by the sequential action of AMP succinate synthetase and AMP succinate lyase [10]. Furthermore, it has been shown that dCK-deficient mutant CEM cells, as well as adenosine kinase-deficient mutant cells, are less capable than wild-type cells of accumulating ddATP from exogenous ddA [9]. Thus, evidence has accrued that suggests that dCK is important for the phosphorylation of various nucleosides of therapeutic interest and is viewed to be of much significance in the design of drugs against HIV. This communication reports on our study of the phosphorylation of the anti-HIV compound (*S,S*)-isoddA by human recombinant dCK.

## MATERIALS AND METHODS

### *Materials*

$Ni^{2+}$–nitrilotriacetic acid–agarose was purchased from Qiagen. Calf liver acetone powder, purine nucleoside phos-

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† *Abbreviations*: AZT, 3′-azido-3′-deoxythymidine; FTC, 5-fluoro-2′,3′-dideoxy-3′-thiacytidine; DTT, dithiothreitol; dCK, 2′-deoxycytidine kinase; and d4T, 3′-deoxy-2′,3′-didehydrothymidine.

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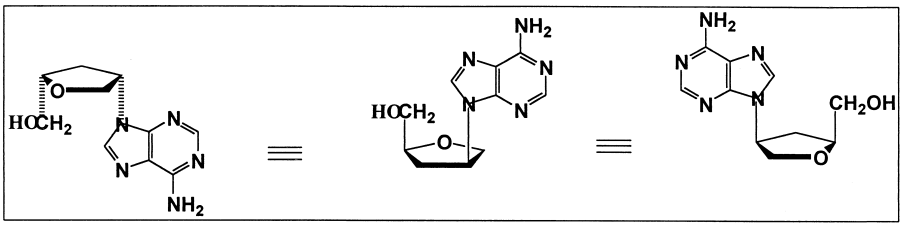

**FIG. 1. Structure of (*S,S*)-isoddA showing isomeric arrangement and absolute stereochemistry.**

phorylase, 5′-AMP–agarose, SDS–PAGE molecular weight marker kit, dC, dCMP, AMP, ADP, and ATP were procured from the Sigma Chemical Co. Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories. Purine nucleoside phosphorylase from calf spleen was purchased from Sigma. Blue-Sepharose was purchased from Pharmacia Biotech AB. The synthesis of (*S,S*)-isoddA has been described [1].

### *Enzyme Preparation and Purification*

Human recombinant dCK cDNA [11] was expressed using the pET 9d bacterial vector as described by Usova and Eriksson [12]. A single colony of bacteria was inoculated in 50 mL M9ZB with antibiotics (50 μM kanamycin and 50 μM chloramphenicol) and was grown overnight with shaking at 37°. The overnight culture (10 mL) was further inoculated into 1000 mL M9ZB with antibiotics (50 μM kanamycin and 50 μM chloramphenicol) and was grown with shaking at 37°. The expression system of the clone bacteria was induced by the addition of isopropyl thio-β-D-galactoside at 1 mM final concentration, and the growth was continued for another 4 hr at 37° with shaking. The cells were harvested by centrifugation at 4500 *g* for 15 min at 4°. In a typical enzyme preparation, bacterial cells (30 g) were incubated first for 30 min at ice bath temperatures using 1 mg/mL of lysozyme in 20 mL of 20 mM Tris–Cl buffer (pH 8.0), 0.5 mM NaCl, 80 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (buffer A) along with the protease inhibitors aprotinin, leupeptin, and pepstatin (prepared according to the protocol of the Qiagen manual). The cells were broken further using a French press. The lysate was centrifuged at 48,000 *g* for 1 hr at 4°. The supernatant was loaded onto a metal chelate affinity column. After washing unbound protein from the column with buffer A, dCK was eluted using 0.5 M imidazole in buffer A and concentrated. DTT (10 mM) and glycerol (20%) were added (for stabilization), and the enzyme solution was concentrated further. A total of 21 mg protein in 4 mL was collected from a typical enzyme purification procedure. The purity of enzyme was established by SDS–PAGE as described by Laemmli [13] using bovine albumin (66 kDa), ovalbumin (45 kDa), pepsin (35 kDa), trypsinogen (24 kDa), and lysozyme (14.3 kDa) as standard protein markers. The enzyme exhibited single-band purity at 33 kDa and also showed a very faint band at about 67 kDa. This faint band of 67 kDa appears to be the dimeric form of the enzyme. Usova and Eriksson [12] reported a similar type of migrating pattern with dCK on SDS–PAGE.

### *Purification of Adenosine Kinase from Calf Liver Acetone Powder*

The purification was carried out by the method of Elalaoui *et al.* [14] except that we used Blue-Sepharose chromatography prior to the 5′-AMP–Sepharose step. The isolated enzyme was stored in the presence of 10% glycerol and 1 mM DTT. The activity of the final preparation was 35.4 μmol/min with a specific activity of 8.4 μmol/min/mg. The purity of the enzyme was checked by SDS–PAGE according to the method of Laemmli [13]. The enzyme preparation exhibited a major band on SDS–PAGE at about 44 kDa. The monomeric form of adenosine kinase has a molecular mass of about 41 kDa [15–17]. The enzyme preparation was free of purine-metabolizing enzymes such as 5′-nucleotidase, adenosine deaminase, and dCK that could interfere in assays involving adenosine kinase.

### *dCK Assay*

In the standard assay mixtures, 5 mM ATP and various concentrations of substrates (e.g. ddA and isoddA) were dissolved in 1 mL of 50 mM Tris–Cl buffer (pH 7.9) containing 150 mM KCl, 5 mM $MgCl_2$, 2 mM DTT. The reaction was initiated by the addition of 100 μL (500 μg) of stock enzyme at 25°. After 60 min, the reaction mixture was analyzed by HPLC using a Particil-10 SAX column (250 mm × 9.4 mm). The mobile phases, which were (A) 100% water, and (B) 0.2 M $KH_2PO_4$ (pH 3.0), 0.8 M KCl, 0.05 M $MgCl_2$, 5% acetonitrile, were used as follows: 0–20 min, 100% A; 20–55 min, 0–52% A/B mixture (linear gradient); 55–75 min, 52–100% A/B mixture (linear gradient); 75–120 min, 100% B (isocratic gradient) [18]. The flow rate was 0.8 mL/min. Identification of products was achieved through comparison of retention times and UV data of authentic samples with those of eluted compounds. The data for substrate activities were fitted into a Michaelis–Menten equation using a computer program utilizing least-squares analysis. Lineweaver–Burk plots yielded the apparent kinetic parameters.

### *Adenosine Kinase Assay with Adenosine as Substrate in a Coupled Enzyme Assay*

Adenosine kinase activity was measured at 25° for 5 min in 50 mM Tris–Cl buffer (pH 7.4), containing 0.1 M KCl, 5 mM $MgCl_2$, 1 mM DTT, 200 μM adenosine, 1 mM ATP, 5 U/mL of pyruvate, 13.8 U/mL of lactate dehydrogenase, 0.5 mM phosphoenolpyruvate, 0.1 mM NADH in a final volume of 1 mL. The reaction was initiated by adding 100 μL (12 μg) of adenosine kinase [16]. NADH oxidation was measured by the decrease in absorbance at 340 nm ($\Delta\epsilon = -6220\ M^{-1}\ cm^{-1}$).

### *Adenosine Kinase Assay*

A typical assay had a final volume of 1 mL of 50 mM Tris-Cl buffer (pH 7.4) containing 0.1 M KCl, 5 mM $MgCl_2$, 1 mM DTT, a 1 mM concentration of substrates (e.g. ddA and isoddA), and 2 mM ATP. The reaction was initiated by adding 100 μL (200 μg) of protein at 25° for 60 min. Product analyses were carried out by HPLC as described in the dCK assay.

### *Purine Nucleoside Phosphorylase Assay*

Experiments were performed at 25° for 60 min in 25 mM Tris–succinic acid buffer (pH 7.0) containing 10 mM phosphate using a 1 mM concentration of substrates (e.g. adenosine, dA, ddA, and isoddA). The reactions were initiated by adding 5 U of enzyme in the case of adenosine and dA and 100 U of enzyme in the case of ddA and isoddA. The reaction mixtures were analyzed by HPLC on an analytical Delta-pak C-18 column (3.8 × 300 mm) with isocratic elution with 5 mM phosphate buffer (pH 4.6) containing 10% methanol [19].

### *Other Enzymes*

5′-Nucleotidase [20] and adenosine deaminase [2, 21] activities were measured using 1 mM IMP and 200 μM adenosine as substrates, respectively, to check for contamination by these enzymes in the adenosine kinase purification.

## RESULTS AND DISCUSSION

dCK was purified to single band homogeneity using $Ni^{2+}$–nitrilotriacetic acid–agarose affinity chromatography [12]. This enzyme was found to phosphorylate isoddA, an anti-HIV active compound, to isoddAMP with a catalytic efficiency of 0.33 nmol/min/mg/μM (Table 1). The catalytic efficiency was markedly less than for dA as substrate, but in the general range for that observed by us for ddA as substrate. The $K_m$ values obtained suggested that ddA has a higher affinity for the enzyme than does its isomeric counterpart, isoddA.

(*S*,*S*)-IsoddA has been shown to inhibit the replication of HIV in different cell lines [2]. An *in vitro* anabolism study of isoddA [2] showed that the concentrations of isoddA in the medium and inside the cells were approximately equal, indicating that the low level of phosphorylation was not due to inefficient transport. The relatively inefficient phosphorylation of this nucleoside may be a limiting factor in its clinical application. Therefore, it was important to examine the structural features necessary for efficient substrate activity. It was found earlier that the catalytic efficiency for phosphorylation of ddC with normal human thymus dCK is about 0.4% of that for dC, although both substrates have the same base moiety [4]. This lower catalytic efficiency may be due to the absence in ddC of the exocyclic 3′-hydroxyl (or 3′-oxygen), which appears to be a requirement for efficient substrate activity. This requirement for an exocyclic oxygen apparently cannot be overcome by an endocyclic oxygen such as is found in (*S*,*S*)-isoddA, which has some of the structural characteristics of a carbocyclic dideoxynucleoside but with an endocyclic oxygen at the 3′-position (Fig. 1). In addition, unlike β-D-dC, (*S*,*S*)-isoddA is related configurationally to the non-natural L-family [1]. It was also discovered that dA and ddA exhibited 15 and 0.2%, respectively, of the activity of dC with human thymus dCK [4], which suggests that there is also a substantial substrate preference for the base cytosine. Thus, the lower catalytic efficiency of (*S*,*S*)-isoddA may be attributed to the presence of a purine base and the regiochemical and configurational modifications of the ribosyl moiety, which result in binding and turnover involving the enzyme in a much less productive way. What is interesting, then, is the observation that the phosphorylation of (*S*,*S*)-isoddA with recombinant dCK (Table 1) is in the general range of that found with β-D-ddA. Unfortunately, the crystal structure of dCK has not been determined, and thus the mode of binding of substrates to this enzyme is not understood completely.

**TABLE 1. Substrate specificity studies with human recombinant dCK**

| Substrate | dCK Activity* $K_m$ (μM) | $V_{max}$ (nmol/min/mg) | $V_{max}/K_m$ (nmol/min/mg/μM) |
|---|---|---|---|
| ddA | 237 | 137 | 0.58 |
| IsoddA | 603 | 200 | .033 |
| dA† | 110 | 800 | 7.3 |
| dC† | 1.5 | 185 | 123.3 |

Substrate activities with dCK were determined as described under Materials and Methods. Kinetic data were calculated from two duplicate experiments.

*$K_m$ and $V_{max}$ values were determined by linear regression analysis.

†Data from Ref. 5.

The question of whether isoddA is initially phosphorylated by other enzymes such as adenosine kinase and 5′-nucleotidase also was examined. As previously shown, ddA is a very poor substrate of adenosine kinase from human lymphoid cells [10]. In this study, no phosphorylated products were observed with ddA and isoddA using purified calf liver adenosine kinase under the assay condi-

**TABLE 2. Substrate behavior of ddA and isoddA toward calf liver adenosine kinase and calf spleen purine nucleoside phosphorylase**

| Substrate | Adenosine deaminase* | Adenosine kinase† | PNPase† |
|---|---|---|---|
| ddA | $K_m$ = 160 μM<br>$V_{max}$ = 0.8 μmol/min | NS | NS |
| IsoddA | $K_m$ = 250 μM<br>$V_{max}$ = $7.8 \times 10^{-5}$ μmol/min | NS | NS |

*Kinetic parameters for adenosine deaminase were determined by Nair *et al.* [2].
†Adenosine kinase and PNPase activities were determined using 1 mM ddA or isoddA and 2 mM ATP as substrates with 200 μg of enzyme as described in Materials and Methods. Each experiment was performed three times. NS = non-substrate.

tions described under Materials and Methods (see also Table 2). Lack of activation of other anti-HIV dideoxynucleosides by adenosine kinase has been documented [6]. Also, there was no phosphorolysis of these substrates using purine nucleoside phosphorylase from calf spleen (Table 2). IsoddA is a very poor substrate of adenosine deaminase, with a catalytic efficiency ($V_{max}/K_m$) that is 0.0008% of that for adenosine [2]. ddA is known to be a relatively good substrate of adenosine deaminase, and its conversion to ddAMP is largely through ddI, which is a substrate for 5′-nucleotidase [10, 22]. However, this route through 5′-nucleotidase phosphorylation cannot activate isoddA because it is a very poor substrate for adenosine deaminase.

In conclusion, the anti-HIV potency of (*S*,*S*)-isoddA results from its conversion to its triphosphate, (*S*,*S*)-isoddATP, for which the critical step appears to be the initial monophosphorylation by dCK. However, the low efficiency of this phosphorylation is a key factor that limits the capacity of human lymphocytes to make (*S*,*S*)-isoddA an exceptionally active anti-HIV agent. The design of compounds to circumvent this first phosphorylation step is in progress.

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