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ENZYMATIC PHOSPHORYLATION AND PYROPHOSPHORYLATION OF 2′,3′-DIDEOXYADENOSINE-5′-MONOPHOSPHATE, A KEY METABOLITE IN THE PATHWAY FOR ACTIVATION OF THE ANTI-HIV (HUMAN IMMUNODEFICIENCY VIRUS) AGENT 2′,3′-DIDEOXYINOSINE

JEAN-FRANÇOIS NAVÉ,*† ANNE ESCHBACH,* DOMINIQUE WOLFF-KUGEL,* SERGE HALAZY* and JAN BALZARINI‡

*Marion Merrell Dow Research Institute, 16 rue d'Ankara, 67000 Strasbourg, France; and ‡Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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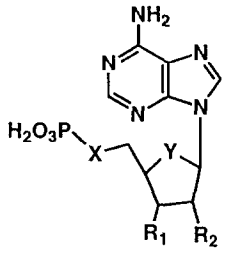
Abstract—2′,3′-Dideoxyadenosine-5′-monophosphate (ddAMP) is a key intermediate in the metabolic pathway involved in the activation of the anti-retroviral agent 2′,3′-dideoxyinosine (ddI) to 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP). The potential phosphorylation of ddAMP by adenylate kinase (myokinase) and pyrophosphorylation by the reverse reaction of 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase were investigated. Using ATP as phosphate donor, ddAMP was phosphorylated by adenylate kinase with an efficiency of 8.8% of that for AMP, as estimated from the V_{max}/K_m ratios. In the presence of PRPP, *Escherichia coli* and rat PRPP synthetases catalysed the pyrophosphorylation of ddAMP with efficiencies of 52 and 35% of that determined for AMP, respectively. Two carbocyclic phosphonate analogues of ddAMP were not substrates of adenylate kinase. Yet, they were pyrophosphorylated by both PRPP synthetases, albeit less efficiently than ddAMP. *In vivo*, the usual function of PRPP synthetase is to synthesize PRPP from ribose-5-phosphate and ATP. In the forward reaction ddATP proved to be a substrate as efficient as ATP for rat PRPP synthetase. ddATP was also studied as a potential phosphate donor in the reaction catalysed by adenylate kinase with AMP as phosphate acceptor and found to be as efficient as ATP. The relevance of these *in vitro* results to the *in vivo* situation is discussed.

Key words: ddAMP; ddATP; adenylate kinase; 5-phosphoribosyl-1-pyrophosphate synthetase; phosphorylation; nucleotide analogues

The nucleoside analogue ddI§ inhibits the replication of HIV, the etiologic agent of AIDS with a favourable therapeutic index [1]. ddI has recently been approved for clinical use by the American Food and Drug Administration. The antiviral activity of ddI is thought to be mediated by inhibition of HIV reverse transcriptase in virus-infected cells [1]. Studies of ddI metabolism in human lymphoid cells have revealed that this compound exerts its antiviral activity by virtue of its ability to generate ddATP [2–4]. ddATP acts as a potent inhibitor of HIV reverse transcriptase [5, 6]. In the metabolic pathway involved in the conversion of ddI to ddATP, ddAMP has been identified as a key intermediate [2]. The enzymatic steps for ddI conversion to ddAMP have been well characterized [2–4]. Yet, the enzymes involved in the conversion of ddAMP to ddATP have not been identified. It is generally assumed that nucleotide analogues of AMP such as ddAMP are phosphorylated intracellularly by adenylate kinase using ATP as the phosphate donor. The resulting nucleoside diphosphate analogue would be subsequently phosphorylated by nucleoside diphosphate kinase, again using ATP as the phosphate donor. Recently, several phosphonate analogues of ddAMP have been designed as antiviral agents [7–9]. Some of these compounds are active against HIV [7, 8]. Again, the antiviral activity of these compounds is thought to be due to inhibition of reverse transcriptase by their diphosphate derivatives (nucleoside triphosphate analogues). The efficiency of conversion of such nucleotide analogues to their diphosphate derivatives by cellular kinases is a critical factor in antiviral activity since it determines the level of their active metabolites in cells. In this respect, a better knowledge of the substrate specificity of the enzymes of the adenylate kinase family, i.e. the cytosolic AMP-K-1 (myokinase) and the mitochondrial AMP-K-2 and AMP-K-3, might help in the discovery of novel and more effective antiviral agents. In a first part of this work, we have investigated and compared the substrate

† Corresponding author. Tel. 88 41 46 05; FAX 88 45 90 70.

§ Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; ddAMP, ddADP and ddATP, 2′,3′-dideoxyadenosine-5′-mono-, 5′-di- and 5′-triphosphate; ddI, 2′,3′-dideoxyinosine; PEP, phosphoenolpyruvate; Rib-5-P, ribose-5-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; AMP-K, adenylate kinase; PK, pyruvate kinase; LDH, lactate dehydrogenase.



Compound	X	Y	R_1	R_2
ddAMP	O	O	H	H
1	CH_2	CH_2	H	H
2	CH_2	CH_2	H	OH
3 *	CH_2	O	OH	OH

* described in reference 27

Fig. 1. Structures of various AMP analogues.

specificity of myokinase (EC 2.7.4.3) for AMP, dAMP, ddAMP and two carbocyclic phosphonate analogues of ddAMP.

In 1984, Sabina *et al.* [10] reported that 5-amino - 4 - imidazolecarboxamide riboside-5′-monophosphate (ZMP), an intermediate of the *de novo* biosynthesis of purines which shares structural similarities with AMP, can be converted directly to its diphosphate derivative ZTP by the reverse reaction of PRPP synthetase although the usual cellular function of PRPP synthetase is to synthesize PRPP from ATP and ribose-5-phosphate [11, 12]. It was suggested that the reverse reaction (conversion of ZMP to ZTP) could be physiologically relevant [10]. More recently, Balzarini and De Clercq [13] reported that 9-β-D-arabinofuranosyladenine-5′-monophosphate, the 5′-monophosphate derivative of the antiherpetic agent ara-A, is converted to its diphosphate derivative ara-ATP by *Escherichia coli* PRPP synthetase in the presence of PRPP. These authors also suggested that PRPP synthetase might be responsible for the cellular pyrophosphorylation of acyclonucleotide analogues such as the antiviral agents 9-(2-phosphonylmethoxyethyl)adenine (PMEA), 9-[(2*S*)-3-hydroxy-2-phosphonylmethoxypropyl]adenine ((*S*)-HPMPA) and 9-[(2*S*)-3-fluoro-2-phosphonylmethoxypropyl]adenine ((*S*)-FPMPA) [14, 15]. These findings prompted us to study ddAMP and two carbocyclic phosphonate analogues of ddAMP as potential substrates of the reverse reaction of rat and *E. coli* PRPP synthetases (EC 2.7.6.1).

We report here the results of these studies which give additional insight into the substrate specificity of the AMP binding sites of adenylate kinase and PRPP synthetase.

MATERIALS AND METHODS

Compounds and enzymes. ddAMP was synthesized from 2′,3′-dideoxyadenosine (from Fluka, Buchs, Switzerland) using the phosphorylation method of Tener [16]. After completion of the reaction, ddAMP (61 mg, estimated from HPLC analysis) was purified on a 2.5 × 36 cm DEAE-Sephadex A-25 column eluted with a linear gradient of NH_4HCO_3 (1 L, 10 mM–1 M). NH_4HCO_3 was removed by successive evaporations of water at 40° with a rotatory evaporator. During this step, the pH was maintained at 7.3 to avoid decomposition under acidic conditions. The compound was further purified by HPLC on a semi-preparative column (Lichrospher 100-RP-18 E, 250-10, Merck, at 4 mL per min) under the same conditions as those described further on. After a second DEAE-Sephadex A-25 column and removal of NH_4HCO_3, the ammonium salt of ddAMP was passed on a 1.1 × 5.5 cm AG-50W-X8 column (Na^+ form) to obtain the sodium salt of ddAMP (24 mg). ddADP and ddATP were obtained from Pharmacia (Uppsala, Sweden). All other nucleotides, NADH, PEP, Rib-5-P, PRPP, BSA, PRPP synthetase (*E. coli*), LDH (bovine heart, type III), PK (rabbit muscle, type II) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Recombinant rat PRPP synthetase (isoform 1) was a generous gift from Dr M. Tatibana (Chiba University, Japan) [17]. Adenylate kinase (myokinase from rabbit muscle) was obtained from Boehringer (Mannheim, Germany). The synthesis of compounds **1** and **2** (Fig. 1) was performed as reported in Refs. 18 and 19, respectively. 9-(5-Phosphonopentyl)adenine was prepared from adenine and 5-bromo-1-(diethylphosphono)pentane according to Ref. 20. [^{3}H]-AMP (27 Ci/mmol) was from Dupont-NEN (Boston, MA, U.S.A.).

Measurements of adenylate kinase (myokinase) activity. The enzyme assay was performed according to the method of Rhoads and Lowenstein [21]. ADP, formed from AMP and ATP, was measured by coupling the myokinase reaction to the PK and LDH reactions. The standard reaction mixture contained 0.1 M triethanolamine buffer (pH 7.6), 0.05% BSA, 10 mM $MgCl_2$, 75 mM KCl, 5 mM dithiothreitol, 1.5 mM PEP, 0.15 mM NADH, 5 U PK/mL, 6.6 U LDH/mL, 1 mM ATP, 0–1 mM AMP and 0.01 μg of myokinase in 1 mL. The reaction was performed at 25° and monitored by measurement of NADH consumption at 340 nm with a DU-7 spectrophotometer. Myokinase was diluted in 0.1 M triethanolamine buffer (pH 7.6) containing 0.05% BSA and 5 mM dithiothreitol, which ensured good stability for at least 4 hr. Solutions of substrates were prepared in 5 mM triethanolamine buffer (pH 7.6) and brought to pH 7.6 with NaOH or HCl.

The phosphorylation of 1 μmol of AMP in the presence of ATP ultimately results in the oxidation of 2 μmol of NADH, since 2 μmol of ADP are produced which serve as substrates for pyruvate kinase. The phosphorylation of dAMP by myokinase

in the presence of ATP leads to formation of dADP and ADP. Similarly, the phosphorylation of ddAMP in the presence of ATP yields ddADP and ADP. To determine the relationship between dAMP or ddAMP phosphorylation and NADH consumption in the myokinase assay, dADP and ddADP were studied as substrates of PK. The PK assay was performed under conditions similar to those used for the myokinase assay, except that myokinase and ATP were omitted and the concentration of PK fixed at 0.016, 0.048 and 0.16 U/mL for evaluation of the kinetic parameters of ADP, dADP and ddADP, respectively. For ADP, dADP and ddADP, K_m values of 0.26, 2.7 and 8.7 mM and V_{max}/K_m values (expressed in per cent of that of ADP) of 100, 3.9 and 0.25 were determined, indicating that dADP is a relatively good substrate of PK whereas ddADP is a poor substrate. These results are in agreement with experiments where the myokinase assay was performed at a concentration of myokinase 250 times higher than that used in the standard assay. In such experiments, addition of e.g. 25 nmol dAMP in the assay led to consumption of 50 nmol NADH before the absorbance reached a constant value. The curve of NADH consumption versus time was exponential. In contrast, when 25 nmol ddAMP were added to the assay mixture, NADH consumption versus time took place in two steps. After an initial decrease of absorbance for 3–4 min corresponding to the consumption of about 25 nmol NADH (phosphorylation of 25 nmol ADP), a slow consumption of NADH was observed over a few hours corresponding to the slow phosphorylation of 25 nmol ddADP by PK. The rate of NADH consumption at the beginning of the second phase represented 3.5% that of the first phase. In conclusion, in the initial velocity measurements, it is assumed that phosphorylation by myokinase of 1 μmol of dAMP results in the oxidation of 2 μmol of NADH whereas for 1 μmol of ddAMP phosphorylated, 1 μmol of NADH is oxidized. In experiments where ddATP is studied as the phosphate donor in the reaction with AMP, ddADP and ADP are the products of the myokinase reaction and it is also assumed that phosphorylation of 1 μmol of AMP leads to oxidation of 1 μmol of NADH. The myokinase activity is expressed in micromoles of mononucleotide phosphorylated per minute per milligram protein. For detection of the potential substrate activity of the carbocyclic phosphonate analogues of ddAMP, the myokinase concentration in the assay was increased as indicated and duplicate assays with or without myokinase were performed. All saturation kinetics done with the various nucleoside mono- and triphosphate substrates followed the Michaelis–Menten equation.

Measurements of PRPP synthetase activity (forward reaction). A spectrophotometric assay based on the method of Valentine and Kurschner [22] was set up. AMP formed from Rib-5-P and ATP was measured by coupling the PRPP synthetase reaction to AMP-K, PK and LDH reactions. In this assay, the pyrophosphorylation of 1 μmol Rib-5-P results in the oxidation of 2 μmol NADH. The reaction mixture contained 50 mM HEPES buffer (pH 7.8), 0.1% BSA, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 50 mM potassium phosphate, 4 mM $MgCl_2$, 1 mM ATP, 0.5 mM Rib-5-P, 0.15 mM NADH, 1.5 mM PEP, 0.83 U AMP-K (myokinase), 1.25 U PK, 1.65 U LDH and 0.17 μg rat PRPP synthetase in a total volume of 0.5 mL. The reaction was performed at 37° and monitored by measurement of NADH consumption at 340 nm. Before use, AMP-K, PK and LDH were dialysed as a concentrated mixture (80-fold with respect to assay concentration) against 20 mM HEPES buffer (pH 7.4) and stored at −20°. The enzyme dilutions were made in a stabilizing solution composed of 50 mM potassium phosphate (pH 7.4), 6 mM $MgCl_2$, 0.1 mM EDTA, 0.3 mM ATP, 2.5 mM 2-mercaptoethanol and 0.1% BSA. Under these conditions, the enzyme remained stable for at least 5 hr. For the determination of the K_m of ATP, ATP was omitted from the enzyme stabilizing solution. The K_m of ATP was determined in the presence of 0.5 mM Rib-5-P and that of Rib-5-P in the presence of 1 mM ATP. At 1 mM ATP and at 0.02 or 0.2 mM Rib-5-P, the initial velocity of the reaction was linear with protein concentration up to at least 0.33 μg enzyme per assay. Enzyme activity is expressed in μmol of Rib-5-P pyrophosphorylated per minute per milligram of protein.

$$\text{Rib-5-P} + \text{ATP} \rightleftharpoons \text{PRPP} + \text{AMP}$$

$$\text{AMP} + \text{ATP} \rightleftharpoons 2\,\text{ADP}$$

$$2\,\text{PEP} + 2\,\text{ADP} \rightleftharpoons 2\,\text{pyruvate} + 2\,\text{ATP}$$

$$2\,\text{pyruvate} + 2\,\text{NADH} \rightleftharpoons 2\,\text{lactate} + 2\,\text{NAD}^+$$

For determination of the kinetic parameters of ddATP, an HPLC method was used. The assay mixture contained 50 mM HEPES buffer (pH 7.8), 0.1% BSA, 1 mM EDTA, 1 mM dithiothreitol, 50 mM potassium phosphate, 4 mM $MgCl_2$, 0.5 mM Rib-5-P, 0–0.2 mM ddATP and 0.06 μg rat PRPP synthetase in a total volume of 100 μL. The reaction was performed at 37°. The enzyme dilutions were made in the above described stabilizing solution where ATP was omitted. The reaction was stopped by 150 μL ice-cold methanol. Preparation of samples for HPLC and analysis of their ddAMP content were performed as described below. ddAMP had a retention time of 6.5 min.

Measurements of PRPP synthetase activity (reverse reaction). The reverse *E. coli* PRPP synthetase reaction was performed at 37° in 50 mM triethanolamine buffer (pH 8.0), containing 0.1% BSA, 10 mM potassium phosphate, 5 mM $MgCl_2$, 2.5 mM PRPP, 0–2 mM AMP and 0.4 μg enzyme in a total volume of 100 μL. The enzyme dilutions and stock solutions of AMP and of the ddAMP analogues were made in 50 mM triethanolamine buffer (pH 8.0) containing 0.1% BSA and 10 mM potassium phosphate and the pH adjusted to 8.0. For the study of the ddAMP analogues as substrates, the enzyme concentration in the assay was increased as indicated. Incubation was stopped by addition of 150 μL of ice-cold methanol and proteins were removed by centrifugation at 10,000 *g* for 4 min. After evaporation under nitrogen, the samples were resuspended in 150 μL HPLC buffer A containing 2%

Table 1. Kinetic parameters of AMP, dAMP and ddAMP for myokinase using ATP as phosphate donor

Compound	K_m (mM)	V_{max}* (% rel. to AMP)	V_{max}/K_m (% rel. to AMP)
AMP	0.11	100	100
dAMP	3.7	64	1.9
ddAMP†	1.4	112	8.8
1‡		No substrate activity at 1 mM	
2‡		No substrate activity at 7 mM	

Data are means of at least two separate determinations, and standard deviations on K_m and V_{max} did not exceed 18 and 11%, respectively.

* V_{max} of AMP = 373 μmol AMP phosphorylated/min/mg protein.

† The kinetic parameters were determined at an enzyme concentration two times higher than that used for AMP and dAMP.

‡ Tested at an enzyme concentration 20 times higher than that used for AMP.

acetonitrile. The nucleoside triphosphate analogues were analysed by HPLC (Lichrospher column 100 RP-18E, 250-4, Merck at 40°; buffer A: 0.1 M KH_2PO_4, 5 mM tetrabutyl ammonium dihydrogen phosphate, pH 5.0; buffer B: buffer A at pH 5.5 containing 40% acetonitrile). A linear gradient (20–44% B in 24 min) was used. Substrates and products of the reaction were detected at 254 nm (Kontron UV 430 detector) and quantified using a titrated ATP solution as reference. The retention times of the diphosphate derivatives of AMP, dAMP, ddAMP, compounds **1** and **2** and 9-(5-phosphonopentyl)adenine were 15.3, 17.4, 17.5, 22.0, 15.1 and 17.6 min, respectively.

The reverse rat PRPP synthetase reaction was performed at 37° in a mixture of 20 mM HEPES and 25 mM triethanolamine buffers (pH 7.9), containing 0.1% BSA, 0.4 mM EDTA, 1 mM dithiothreitol, 60 mM potassium phosphate, 4.6 mM $MgCl_2$, 2 mM PRPP, 0–4 mM AMP and 0.011 μg enzyme in a total volume of 100 μL. For study of the ddAMP analogues as substrates, the enzyme concentration in the assay was increased as indicated. Stock solutions of substrates were the same as those used for study of the *E. coli* enzyme. Enzyme dilutions were made in the above described stabilizing buffer without ATP. In this buffer at 0°, the enzyme was stable for at least 4 hr. The reaction was stopped and the samples analysed by HPLC as described above for the *E. coli* enzyme assay.

In both the *E. coli* and rat PRPP synthetase assays, the formation of products was linear with time for all substrates studied. The saturation kinetics of substrates for both enzymes followed the Michaelis–Menten equation.

The reverse rat PRPP synthetase radiochemical assay was performed in 50 mM HEPES buffer (pH 7.7), 0.1% BSA, 1 mM EDTA, 1 mM dithiothreitol, 50 mM potassium phosphate, 4 mM $MgCl_2$, 2 mM PRPP, 0.4 μCi[^{3}H]AMP, 0–2 mM AMP and 0.07 μg enzyme in a total volume of 100 μL. After incubation for 10 and 20 min at 37°, aliquots (10 μL) were spotted on a PEI (polyethyleneimine) cellulose plastic sheet and developed with a 0.3 M LiCl–0.5 M formic acid solution by ascending chromatography. The band containing [^{3}H]ATP was extracted for 10 min in 2 mL of 2 M ammonium formate–16 M formic acid solution and radioactivity was counted in 10 mL aquasol-2 (Dupont-NEN). The reaction rate was linear with time and amount of enzyme under these conditions. The K_m of PRPP was determined under similar experimental conditions. Saturation kinetics of the enzyme by AMP (0–2 mM) were performed at various fixed concentrations of PRPP (0.05–1 mM). Double reciprocal plots of apparent maximal velocities versus PRPP concentrations were used for determination of the K_m of PRPP.

Miscellaneous methods. Kinetic parameters of substrates were determined using a non-linear regression analysis program Enzfitter (Biosoft).

RESULTS

Study of ddAMP and carbocyclic phosphonate analogues of ddAMP as substrates of myokinase

ddAMP was easily phosphorylated by myokinase. From saturation kinetics, a V_{max} value slightly higher than that of the natural substrate AMP was found (Table 1). However, the K_m value of ddAMP was approx. 13-fold higher than that of AMP. Considering the V_{max}/K_m ratios, ddAMP was phosphorylated with an efficiency of 8.8% of that for AMP. Hence, ddAMP is a relatively good substrate of myokinase. Moreover, ddAMP was also found to be a 4–5-fold better substrate of myokinase than dAMP (Table 1). The carbocyclic phosphonate analogues of ddAMP (compound **1**, Fig. 1) and 3′-deoxyadenosine-5′-monophosphate (compound **2**, Fig. 1), tested at concentrations of 1 and 7 mM, respectively, were not phosphorylated by myokinase when the myokinase concentration was 20-fold higher than that used for AMP (Table 1). At this high concentration of enzyme, it was possible to detect the substrate activity of compounds phosphorylated at a rate corresponding to 0.2% of the maximal velocity calculated for AMP at the same enzyme concentration.

Comparison of ATP and ddATP as substrates of myokinase

Saturation kinetics of myokinase by AMP (varied

Table 2. Comparison of the kinetic parameters* of the forward and the reverse reactions of rat PRPP synthetase

Substrates	V_{max} (μmol/min/mg)	K_m of substrates (μM)	Assay
Forward reaction			
ATP and Rib-5-P	19.4 ± 0.8 (N = 6)	23 ± 0 (N = 2) (ATP) 54 ± 11 (N = 5) (Rib-5-P)	Spectrophotometric
Reverse reaction			
AMP and PRPP	4.3 ± 0.5 (N = 10)	250 ± 60 (N = 12) (AMP)	HPLC
	4.9 ± 0.7 (N = 3)	310 ± 50 (N = 3) (AMP) 110 (PRPP)	Radiochemical

* Results are presented as means ± SD (number of determinations).

substrate) at various fixed concentrations of ATP allowed the determination of a series of apparent maximal velocities (data not shown). The Michaelis constant of ATP at saturating concentration of AMP was then determined from double reciprocal plots of the apparent maximal velocities versus the concentration of ATP. A K_m of 73 μM was found for ATP. Using the same approach, a K_m of 85 μM was determined for ddATP. The V_{max} values for AMP phosphorylation using ATP or ddATP as phosphate donor were similar (379 and 406 μmol AMP phosphorylated/min/mg protein, respectively). Considering the V_{max}/K_m ratios, ddATP was a phosphate donor as efficient as ATP for the myokinase reaction.

Comparison of the forward and reverse reactions of rat PRPP synthetase

The kinetic parameters of the forward (PRPP synthesis) and reverse (PRPP consumption) reactions of rat PRPP synthetase (recombinant enzyme, isoform I obtained from Ishijima *et al.* [17]) were investigated at a potassium phosphate concentration of 50 mM. The results are summarized in Table 2. For the forward reaction, a specific activity of 19.4 μmol Rib-5-P pyrophosphorylated/min/mg protein was found. This value is in good agreement with the value of 25.2 μmol/min/mg protein determined by Tatibana (personal communication) for the same batch of enzyme using an assay measuring the Rib-5-P-dependent [^{14}C]AMP production from [^{14}C]ATP at a potassium phosphate concentration of 10 mM under otherwise similar conditions [23]. For the Michaelis constants of ATP and Rib-5-P, values of 23 and 54 μM were obtained (Table 2), which again are close to those previously reported for this enzyme [17]. The reverse reaction was studied using two different methods which gave similar results. The maximal velocity of the reverse PRPP synthetase reaction was approx. 4-fold lower than that of the forward reaction (Table 2). For the human erythrocyte enzyme under similar experimental conditions, the V_{max} for the reverse reaction was reported to be 7-fold [10] and 3.8-fold [24] lower than that of the forward reaction. The K_m value for AMP in the reverse PRPP synthetase reaction was approx. 12-fold higher than that of ATP in the forward reaction.

Pyrophosphorylation of ddAMP and carbocyclic phosphonate analogues of ddAMP by rat and E. coli *PRPP synthetases (reverse PRPP synthetase reaction)*

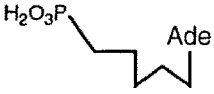
In the presence of enzyme-saturating concentrations of PRPP, dAMP and ddAMP were efficiently pyrophosphorylated by rat PRPP synthetase. The efficiencies of pyrophosphorylation (V_{max}/K_m) of dAMP and ddAMP were 32 and 35% that of AMP (Table 3). In contrast, the carbocyclic phosphonate analogue of ddAMP (compound **1**) was a very poor substrate of the rat enzyme since it was 194-fold less efficiently phosphorylated than ddAMP. Compound **2**, the 2'-hydroxy derivative of **1**, was also a poor substrate. Yet, it was a 5-fold more efficient substrate than **1** (Table 3). Unexpectedly, 9-(5-phosphonopentyl)adenine, which can be regarded as an acyclic derivative of the carbocyclic phosphonate **1**, was also pyrophosphorylated by rat PRPP synthetase with an efficiency similar to that of compound **1**.

The substrate specificity of the bacterial PRPP synthetase was also studied using the same set of compounds. dAMP and ddAMP were found to be very efficient substrates of the *E. coli* enzyme (Table 3). Surprisingly, the carbocyclic phosphonates **1** and **2** and 9-(5-phosphonopentyl)adenine were found to be relatively good substrates of *E. coli* PRPP synthetase. The pyrophosphorylation efficiencies for these three compounds were about 10 times higher than those determined with the rat enzyme.

Comparison of ATP and ddATP as substrates of rat PRPP synthetase (forward PRPP synthetase reaction)

Our finding that ddAMP is an efficient substrate of the reverse reaction of PRPP synthetase suggested that ddATP could serve as a substrate of the forward reaction catalysed by the enzyme. It is not possible to evaluate the kinetic parameters of ddATP with the spectrophotometric assay since ddADP formed in the assay is a poor substrate of PK (see Materials and Methods). Therefore, the kinetic parameters of ddATP were determined by the HPLC method (see Materials and Methods). However, the HPLC method was not applicable to the measurement of AMP formation from ATP since the AMP peak could not be separated from a major peak of the chromatogram. In this case, the spectrophotometric

Table 3. Kinetic parameters of AMP analogues for rat and *E. coli* PRPP synthetases (reverse reaction)

Compound	Rat enzyme			*E. coli* enzyme		
	K_m (mM)	V_{max}* (% rel. to AMP)	V_{max}/K_m (% rel. to AMP)	K_m (mM)	V_{max}* (% rel. to AMP)	V_{max}/K_m (% rel. to AMP)
AMP	0.25	100	100	0.29	100	100
dAMP	0.68	88	32	0.49	111	66
ddAMP	0.63	87	35	0.50	90	52
1	1.7	1.2	0.18	0.55	3.0	1.6
2	1.1	3.9	0.89	0.24	7.4	8.9
	1.5	1.5	0.25	0.69	7.5	3.2

Data are means of at least two separate determinations, and standard deviations on K_m and V_{max} did not exceed 24 and 13% for the rat enzyme and 15 and 18% for the *E. coli* enzyme, respectively.

The kinetic parameters of dAMP, ddAMP, of compounds **1**, **2** and of 9-(5-phosphonopentyl)adenine were determined at enzyme concentrations 1-, 2-, 10-, 10- and 10-fold (rat enzyme) and 1-, 1-, 6-, 6- and 6-fold (*E. coli* enzyme) higher than that used for AMP, respectively.

* V_{max} of AMP = 4.3 and 0.58 μmol AMP pyrophosphorylated/min/mg protein for the rat and *E. coli* enzymes, respectively.

assay was used. In the presence of enzyme-saturating concentrations of Rib-5-P (0.5 mM), the maximal velocity determined for ddATP (4.4 μmol/min/mg protein) was approx. 3-fold lower than that of ATP (12.7 μmol/min/mg protein). The K_m of ddATP (7 μM) was also about 3-fold lower than the K_m measured for ATP (23 μM). Considering the V_{max}/K_m values, this means that ddATP is a substrate of rat PRPP synthetase as efficient as ATP.

DISCUSSION

ddAMP has been recognized as a key intermediate in the metabolic pathway by which the antiretroviral agent ddI is converted to the active metabolite ddATP [2]. Yet, the enzyme(s) responsible for the phosphorylation of ddAMP has not been identified. In this work, we show that the isoenzyme AMP-K-1 (myokinase) of the adenylate kinase family catalyses the phosphorylation of ddAMP with a relatively good efficiency and that ddAMP is a better substrate than dAMP (Table 1). Since dAMP has been reported to be a substrate of the adenylate kinase isoenzymes AMP-K-2 [25] and AMP-K-3 [26], ddAMP might also serve as a substrate for these enzymes. Neither the carbocyclic phosphonate analogue of ddAMP (compound **1**, Fig. 1) nor its 2'-hydroxy derivative (compound **2**) are phosphorylated by myokinase, indicating that either the phosphoester bond and/or the oxygen atom of the ribofuranose moiety of AMP are essential for the binding and/or the phosphorylation of AMP by myokinase. In substrate specificity studies with the rabbit muscle adenylate kinase (myokinase), Hampton *et al.* [27] reported that the phosphonate analogue of AMP (compound **3**, Fig. 1) is 118-fold less efficient substrate than AMP. Thus, since we found that ddAMP is 11-fold less efficient substrate of myokinase than AMP, a phosphonate analogue of ddAMP might be a very poor substrate of the enzyme. This could explain why it was not possible to detect phosphorylation of compounds **1** and **2** under our experimental conditions.

The transphosphorylation reactions catalysed by myokinase and other adenylate kinases are readily reversible [28]. *In vivo*, adenylate kinases appear to facilitate high energy phosphoryl transfer between ATP-utilizing and ATP-generating systems and are able to function both in the direction of AMP phosphorylation and ADP consumption [29]. Therefore, adenylate kinases (myokinase and possibly its isoenzymes) might be responsible for the phosphorylation of ddAMP to ddADP in intact cells.

Previous *in vitro* studies have shown that PRPP synthetase is not only able to convert various ATP analogues to the corresponding AMP analogues but also to catalyse the reverse reaction, namely the pyrophosphorylation of the AMP analogues [10, 13, 14, 19]. Several of the AMP analogues which serve as substrates of PRPP synthetase are antiviral agents. Yet, it is currently unclear whether in intact cells these analogues are converted by PRPP synthetase to their antivirally active diphosphate derivatives. The reverse reaction has been studied for the *Salmonella typhimurium* [30], *E. coli* [14] and human erythrocyte [10] enzymes for which Michaelis constants of 0.32, 0.18 and 0.37 mM were determined for AMP. These values are in the same range as those found here for the *E. coli* (0.29 mM) and rat (0.25 mM) enzymes. The ratios of maximal velocities for the forward to the reverse reaction which have been reported previously for the human erythrocyte enzyme (7-fold [10] and 3.8-fold [24]) are also comparable to that found in this study for the rat enzyme (4-fold). dAMP and ddAMP are efficient substrates for both the rat and the *E. coli* enzyme (Table 3). The observation that the kinetic parameters of dAMP and ddAMP are very similar

suggests that the 3′-hydroxyl group of AMP does not play a significant role in binding or catalysis. On the other hand, the 2′-hydroxyl group of AMP must interact with the active site of the enzyme since a significant difference between the K_m values of AMP and dAMP is observed. However, unlike ddAMP, the carbocyclic phosphonate **1** is a poor substrate of rat PRPP synthetase, pointing out the importance of either the phosphoester moiety and/or the oxygen atom of the ribofuranose of AMP both in catalysis and binding. The carbocyclic phosphonate **2**, which is the 2′-hydroxy derivative of **1** is a 5-fold better substrate of the rat enzyme than **1**. Again, this observation points to a role for the 2′-hydroxyl group of AMP in the recognition by the enzyme. The mammalian enzyme appears more stringent in its substrate specificity than the bacterial enzyme. Thus, whereas the carbocyclic phosphonates **1** and **2** are 555- and 112-fold less efficient substrates than AMP for the rat enzyme, they are only 63- and 11-fold less efficient than AMP for the *E. coli* enzyme. The higher specificity of the rat enzyme is also observed with 9-(5-phosphonopentyl)adenine (Table 3).

Our finding that ddAMP is converted to ddATP by mammalian PRPP synthetase with an efficiency only 3-fold lower than that of AMP is remarkable. However, this result was obtained in the presence of enzyme-saturating concentrations of PRPP, i.e. under conditions which are not encountered in intact cells. In cells, PRPP levels range from 5 to 30 μM [11], i.e. below the K_m of PRPP (110 μM) determined for the rat enzyme. Cellular ATP levels are in the millimolar range, i.e. at least 50-fold higher than the K_m of ATP. Such conditions are in favour of the forward PRPP synthetase reaction. The cellular function of PRPP synthetase is to synthesize PRPP from ATP and ribose-5-phosphate [11, 12]. Whether under specific metabolic conditions, PRPP synthetase is able to pyrophosphorylate AMP with consumption of the PRPP pool (reverse reaction) is not known. Therefore, the pyrophosphorylation of ddAMP and related nucleotide analogues by PRPP synthetase in intact cells remains hypothetic.

We also found that ddATP is a phosphate and pyrophosphate donor as efficient as ATP in adenylate kinase- and PRPP synthetase-catalysed reactions, respectively, suggesting that these enzymes might play a role in the catabolism of ddATP. However, in human T-cell lines [31] and in peripheral blood mononuclear cells [32] exposed to ddI concentrations sufficient to inhibit HIV infection (5–10 μM), ddATP levels are approx. 0.05–0.2 μM, i.e. four to five orders of magnitude lower than ATP levels. Therefore, it is unclear whether adenylate kinases and PRPP synthetase play a relevant role in the catabolism of ddATP in intact cells.

In conclusion, considering the usual function of adenylate kinases and PRPP synthetase in intact cells, adenylate kinases (myokinase and possibly its isoenzymes) rather than PRPP synthetase might be involved in the anabolism (phosphorylation) of ddAMP. Both enzymes might contribute to the catabolism of ddATP. The extent to which these two enzymes may contribute in intact cells to the control of the level of ddATP, the active metabolite of ddI, remains to be investigated.

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