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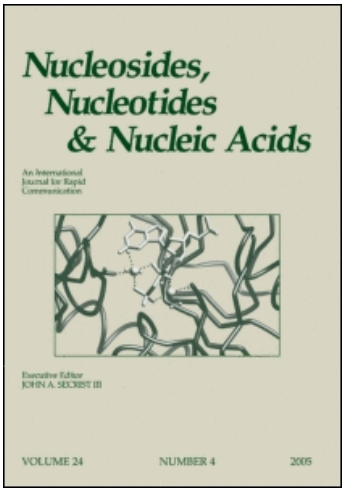

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Kinetic Properties of Adenine Nucleotide Analogues Against Purified 5-Phosphoribosyl-1-pyrophosphate Synthetases from *E. coli*, Rat Liver and Human Erythrocytes

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KINETIC PROPERTIES OF ADENINE NUCLEOTIDE ANALOGUES AGAINST PURIFIED 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE SYNTHETASES FROM *E.COLI*, RAT LIVER AND HUMAN ERYTHROCYTES

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ABSTRACT. The nucleoside analogue 2',3'-dideoxyadenosine (ddA), the phosphonate isostere of 2',3'-dideoxy-2',3'-didehydro-adenosine (d4A) 5'-monophosphate (d4API), and the acyclic nucleoside phosphonates PMEoA, PMEA, FPMPA and PMPA are potent and selective antiretroviral agents. We found that these compounds are recognized as substrates by the PRPP synthetases from *E.coli*, rat liver and human erythrocytes, as their monophosphate and triphosphate form in the reverse and forward reaction, respectively. In particular, ddA-5'-monophosphate (ddAMP) and ddA-5'-triphosphate proved to be excellent substrates for the enzymes. D4API was a relatively good substrate of the rat liver and human erythrocyte PRPP synthetases. The acyclic nucleoside phosphonates were rather poor substrates, as evident from their low V_{max} values. None of the PRPP synthetases are found to act stereospecifically: they recognized both the *S*- and *R*-enantiomers of FPMPA and PMPA in a comparably efficient manner. Our data indicate that PRPP synthetase may recognize a much broader range of adenine nucleotide analogues than previously thought.

INTRODUCTION

5-Phosphoribosyl-1-pyrophosphate synthetase (PRPP-S) is a key enzyme in *de novo* purine and pyrimidine nucleotide synthesis, and reversibly catalyses the transfer of a pyrophosphate group from ATP to the 1-hydroxyl group of ribose-5'-phosphate. The enzyme reaction mainly proceeds in the direction of PRPP formation. PRPP synthetase has been purified from several sources, including the procaryotes *Escherichia coli* (1), *Salmonella typhimurium* (2), and *Bacillus subtilis* (3), and mammalian rat liver (4,5) and human erythrocytes (6). PRPP synthetase is known to display a high substrate specificity. Indeed, besides AMP and ATP, only

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few nucleotides are reported to be recognized by the enzyme as substrates. In 1984, Sabina and co-workers (7) reported the one-step conversion of ZMP (5-amino-4-imidazolecarboxamide riboside 5'-monophosphate) to its diphosphorylated derivative ZTP. Also, tubercidin 5'-monophosphate acts as a substrate in the reverse reaction catalysed by PRPP synthetase (7). Recently, we found that acyclic (purine) nucleoside phosphonate (ANP) derivatives may act as substrates for the PRPP synthetase reaction as well (8-11). The ANP derivatives represent a novel class of synthetic compounds that are potent and selective inhibitors of retroviral [i.e. human immunodeficiency virus (HIV)] and hepatitis B virus infections (10-13) (Fig. 1). Also, araAMP, the 5'-monophosphate of the antiherpetic agent araA (vidarabine, 9-ß-D-arabinofuranosyladenine) was reported to act as a substrate for *E.coli* PRPP synthetase (14). More recently, ddAMP as well as ddATP, an active metabolite of the anti-HIV compounds 2',3'-dideoxyadenosine (ddA) and 2',3'-dideoxyinosine (ddI), were also found to be efficient substrates of rat liver PRPP synthetase (15). We now report on the kinetic properties of a number of antiretrovirally active ANP derivatives, the dideoxynucleotide analogues ddAMP and ddATP and their isosteric phosphonate derivatives d4API and d4APIpp against PRPP synthetase from several sources, including *E.coli*, rat liver and human erythrocytes. We found that the dideoxynucleotide analogues ddAMP and ddATP show marked substrate affinities for all three enzyme sources with efficiencies comparable to that of the natural substrates AMP and ATP. The d4API isostere was a relatively good substrate of the mammalian PRPP synthetases. In contrast, the acyclic nucleoside phosphonates proved markedly less efficient substrates for all PRPP synthetases, as evident from the rather low V_{max}, as compared to that of the natural substrates AMP and ATP.

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MATERIALS AND METHODS

The origin of the test compounds was as follows: adenosine-5'-monophosphate (AMP), adenosine-5'-triphosphate (ATP), 2',3'-dideoxyadenosine-5'-monophosphate (ddAMP) and 2',3'-dideoxyadenosine 5'-triphosphate (ddATP) were from Sigma Chemical Company (St. Louis, MO). The phosphonate isostere of 2',3'-dideoxy-2',3'-didehydro-AMP (d4API) was from Bristol Myers Squibb (Wallingford, CO). The acyclic nucleoside phosphonates 9-(2-phosphonylmethoxyethyl)adenine (PMEA), the (*S*) and (*R*) enantiomers of 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA) and 9-(2-phosphonylmethoxypropyl)adenine (PMPA) were provided by A. Holy (Prague, Czech Republic) and the ethoxy derivative of PMEA (PMEoA) was from Smith-Kline Beecham (Epsom, Surrey, United Kingdom). The diphosphorylated derivatives of PMEA and PMEoA were kindly provided by A. Holy and Smith-Kline Beecham, respectively. The structural formulae of the test compounds are depicted in Fig. 1. The other reagents used were of the highest attainable quality.

The reverse PRPP synthetase reaction mixture (100 µl) contained 10 mM potassium phosphate buffer, pH 8.0, 5 mM $MgCl_2$, 2.5 mM PRPP, an appropriate amount of AMP or test compound, and an appropriate amount of PRPP synthetase. The reaction mixture was then

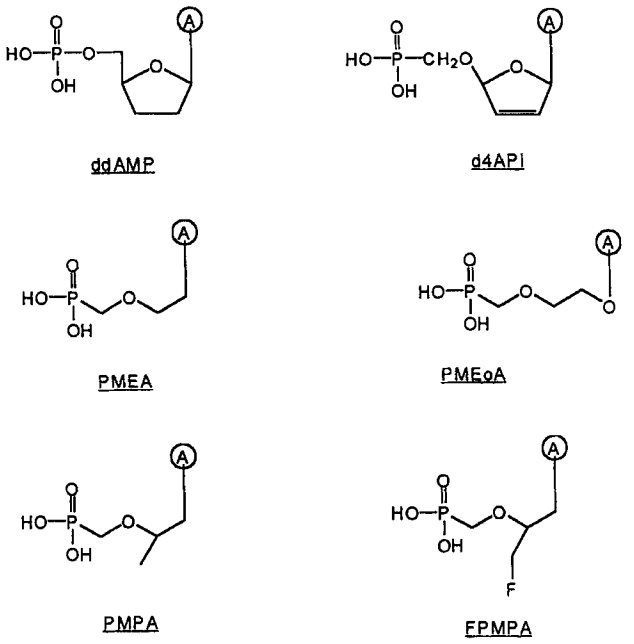

FIG. 1
Structural formulae of adenine derivatives

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incubated at 37°C for 10 min (AMP, ddAMP) or 4 h (d4API and acyclic nucleoside phosphonates). During these incubation times, the reaction proceeded linearly. The assays were terminated by adding methanol at a final concentration of 60% to inactivate and remove the enzyme from the supernatant. The forward PRPP synthetase reaction was performed under similar conditions except that ATP, ddATP, PMEoApp and PMEApp were used as substrates and PRPP was replaced by ribose-5-phosphate (1 mM). The formation of ATP or diphosphorylated test compound in the reverse reaction, or AMP or monophosphorylated test compound in the forward reaction, was analyzed by HPLC on an anion-exchange Partisphere column, and the constituents were separated by a linear gradient of 7 mM $(NH_4)H_2PO_4$ (pH 3.80) to 250 mM $(NH_4)H_2PO_4$ + 500 mM KCl (pH 4.50).

The inhibition of AMP phosphorylation by the test compounds was examined in the presence of different substrate (AMP) concentrations (i.e. 300, 250, 200, 100, 75, and 50 μM) and appropriate concentrations of the test compounds. Enzyme concentration was 0.002 units (*E.coli*) and 0/0012 units (human erythrocytes)/reaction mixture, and reaction time was 10 min. At this enzyme concentration, there was no detectable conversion of the test compounds to their diphosphorylated derivatives. For ddAMP, there was a minor conversion to its ddATP derivative.

E.coli PRPP synthetase was obtained from Sigma Chemical Company. The isolation and purification of the rat liver PRPP synthetases type I and type II and human erythrocyte PRPP synthetase has been previously described (16,17).

RESULTS

Kinetic properties of different PRPP synthetases for adenine nucleotide analogues, as measured in the "reverse" reaction

In our studies, we utilized purified PRPP synthetase from three different sources: *Escherichia coli*, human erythrocytes and rat liver. Of the latter enzyme, the isoenzymes I and II were investigated. The kinetic properties of the enzymes for the natural substrate AMP, 2',3'-dideoxyadenosine 5'-monophosphate (ddAMP), the phosphonate isoster of 2',3'-dideoxy-2',3'-didehydro-adenosine-5'-monophosphate (d4API) and the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and its ethoxy analogue (PMEoA), the (*R*) and (*S*) enantiomers of 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA) and 9-(2-phosphonylmethoxypropyl)adenine (PMPA) were investigated (Fig. 1, Tables 1a and b).

In the presence of 2.5 mM PRPP, the *E.coli* PRPP synthetase catalysed the synthesis of ATP, ddATP, d4APIpp and the diphosphorylated derivatives of PMEA, PMEoA, FPMPA and PMPA. The kinetic parameters of the "reverse" *E.coli* PRPP synthetase reaction with AMP, ddAMP, d4API and the acyclic nucleoside phosphonates are listed in Table 1a.

ddAMP had a K_m value that was only 3-fold higher than that of AMP (377 μM and 132 μM, respectively), whereas d4API (K_m: 2,000 μM) showed a 15-fold lower affinity for the enzyme than AMP. Among the acyclic nucleoside phosphonate derivatives, PMEoA had a 4- to 5-fold higher K_m than the natural substrate, whereas the phosphonylmethoxyethyl (PME), phosphonylmethoxypropyl (PMP) and fluorophosphonylmethoxypropyl (FPMP) derivatives of adenine showed a ≥ 10-fold lower affinity for PRPP synhetase than AMP. The V_{max} value of ddAMP was 2-fold higher than that observed for AMP, whereas d4API conversion by the *E.coli* enzyme proceeded at a 6-fold lower rate than for AMP. The ANP analogues had substantially lower V_{max} values (0.16-0.51 μmole/mg/hr) than the V_{max} for AMP (16 μmole/mg/hr). Consequently, the phosphorylating efficiency of the enzyme was the highest for AMP and ddAMP (V_{max}/K_m: 0.08-0.12), whereas the V_{max}/K_m ratio for the other compounds proved approximately 100-fold (d4API), and 300- to 1,200-fold (ANP analogues) lower than the corresponding V_{max}/K_m ratio for AMP (Table 1a).

The kinetic parameters of the test compounds were also evaluated for PRPP synthetases from rat liver (type I and type II) and human erythrocytes (Table 1b). Comparing K_m and V_{max} values of the test compounds for isoenzymes I and II from rat liver, differences generally lower than 2-fold were observed. Based on V_{max}/K_m ratios, the specificity of the two isoenzymes for the test compounds was quite similar. Again, the best phosphorylating efficiencies were noted

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TABLE 1a
Kinetic properties of PRPP synthetase from *E.coli* for adenine nucleotide analogues

Substrate	*E.coli*[a,b]		
	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m
AMP	132	16	0.12
ddAMP	377	31	0.08
d4API	2,000	2.7	0.001
PMEoA	588	0.24	0.0004
PMEA	1,470	0.21	0.0001
(*S*)PMPA	1,540	0.16	0.0001
(*R*)PMPA	1,290	0.16	0.0001
(*S*)FPMPA	575	0.18	0.0003
(*R*)FPMPA	1,140	0.51	0.0004

[a]The *E.coli* enzyme was used at 0.001 units per assay (100 μl) for AMP (reaction time: 10 min) and ddAMP (reaction time: 20 min), 0.04 units per assay for PMEA, PMEoA and d4API (reaction time: 4 hrs), and 0.1 units per assay for PMPA and FPMPA (reaction time: 4 hrs).
[b]Data are the mean for at least 2 to 4 independent experiments.

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TABLE 1b
Kinetic properties of PRPP synthetase from rat liver and human erythrocytes for adenine nucleotide analogues[a]

	Rat liver[b]						Human erythrocytes[c]		
	Type 1			Type 2					
	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m
AMP	263	119	0.45	167	75	0.45	122	8.8	0.07
ddAMP	323	130	0.40	317	137	0.43	-	-	-
d4API	597	25	0.04	1,579	44	0.028	411	2.0	0.005
PMEoA	308	3.9	0.012	789	9.0	0.011	422	0.32	0.0008
PMEA	656	0.93	0.001	1,013	2.2	0.002	661	0.057	0.0001
(*S*)PMPA	-	-	-	-	-	-	2,174	0.035	0.00002
(*R*)PMPA	-	-	-	-	-	-	3,571	0.056	0.00002
(*S*)FPMPA	3,636	1.9	0.0005	-	-	-	-	-	-
(*R*)FPMPA	1,923	1.2	0.0006	-	-	-	-	-	-

[a]Date are the mean of at least 2 to 4 independent experiments.
[b]The rat liver enzymes were used at 0.005 units per assay (100 μl) for all compounds tested. Reaction time was 10 min and 20 min for AMP and ddAMP, respectively, and 4 hrs for the other compounds.
[c]The human erythrocyte enzyme was used at 0.0012 units per assay (100 μl) for AMP (reaction time: 10 min), and at 0.005 units per assay for the other test compounds.

for AMP and ddAMP (V_{max}/K_m: 0.40-0.45 μmole/mg/hr). Interestingly, in contrast to findings with the *E.coli* enzyme, d4API was found to be a relatively good substrate of rat liver PRPP synthetases I and II, with V_{max}/K_m values respectively 11- and 20-fold lower than for AMP. The phosphorylating efficiency of the rat liver isoenzymes for these compounds was greater than that of the *E.coli* enzyme, as reflected by the higher V_{max} values. Also, PMEA and PMEoA were more efficiently converted to their diphosphorylated derivatives by the rat liver isoenzymes than by the *E.coli* enzyme, as again, reflected by the markedly higher V_{max} values. The (*S*) and (*R*) enantiomers of FPMPA were both poor substrates of the rat liver enzyme (Table 1b).

As a rule, the human erythrocyte enzyme had comparable K_m values, but markedly lower V_{max} values for all test compounds than its rat liver counterparts. Consequently, the phosphorylating efficiency of human erythrocyte PRPP synthetase was 5- to 8-fold lower for AMP and d4API, and 10- to 20-fold lower for PMEA and PMEoA (Table 1b).

In general, none of the enzymes seem to markedly discriminate between the (*S*) and (*R*) enantiomers of the ANP derivatives with regard to both K_m and V_{max} values. However, it should be mentioned that the K_m values of the (S) and (R) enantiomers of PMPA and FPMPA are much larger than those of the natural substrates (including PMEA), so that steric hindrance of the methyl or fluoromethyl group in these molecules cannot be ruled out.

Inhibitory activity of adenine nucleotide analogues against *E.coli* and human erythrocyte PRPP synthetase

ddAMP, d4API, PMEoA and PMEA were also evaluated for their inhibitory effects on *E.coli* and human erythrocyte PRPP synthetases in the presence of the natural substrate AMP (100 μM). The IC_{50} (50% inhibitory concentration) of ddAMP was 0.55-0.93 mM, whereas d4API was not markedly inhibitory at 2.5 mM (Table 2). In contrast, PMEA and PMEoA inhibited AMP phosphorylation by *E.coli* PRPP synthetase at 1.9 and 0.86 mM, respectively, and AMP phosphorylation by human erythrocyte PRPP synthetase at 2.1 and > 2.5 mM (Table 2). The K_i values of ddAMP, d4API, PMEoA and PMEA for *E.coli* PRPP synthetase coincided closely with their IC_{50} values. The Ki values of human erythrocyte enzyme were 2-fold (ddAMP) and 6-fold (PMEA) lower than the IC_{50} values.

Based on the K_i/K_m values, ddAMP had the best affinity for the PRPP synthetases. This is in agreement with the prominent substrate affinity of ddAMP for PRPP synthetase (Table 1a and b). Also, the K_i of the human erythrocyte enzyme for PMEA, and the K_i of the *E.coli* enzyme for PMEA, PMEoA and d4API correlated relatively well with the K_m values of the enzymes (Tables 1 and 2). The type of inhibition was competitive to linear mixed competitive

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TABLE 2
K_i and K_i/K_m values of adenine nucleotide analogues for the conversion of AMP to ATP by *E.coli* and human erythrocyte PRPP synthetase[a]

Compound	PRPP synthetase[b]	IC_{50}[c] (mM)	K_i[d] (mM)	K_i/K_m[e]	Type of inhibition[f]
ddAMP	*E.coli*[g]	0.55	0.55	3.0	C
	H.E.[g]	0.93	0.41	3.9	LM
d4API	*E.coli*	> 2.5	7	49	LM
	H.E.	> 2.5	-	-	-
PMEoA	*E.coli*	0.86	1.4	6.3	LM
	H.E.	> 2.5	-	-	-
PMEA	*E.coli*	1.9	1.9	20	C
	H.E.	2.1	0.37	4.9	LM

[a]Data are the mean of at least 2 to 4 independent experiments.
[b] *E.coli*: *Escherichia coli*; H.E.: human erythrocytes.
[c]IC_{50} or 50% inhibitory concentration in the presence of 100 μM AMP.
[d]K_i values were calculated from Dixon plots derived from the Lineweaver-Burk diagrams.
[e]K_m for AMP was 132 μM for *E.coli* PRPP synthetase and 122 μM for human erythrocyte PRPP synthetase.
[f]C: competitive; LM: linear mixed.
[g]The *E.coli* and human erythrocyte enzymes were used at 0.002 unit and 0.0012 units per assay, respectively. Reaction time was 10 min.

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with respect to AMP as the natural substrate, depending on the nature of the test compound and the enzyme investigated (Table 2).

Kinetic properties of different PRPP synthetases for adenine nucleotide analogues as measured in the "forward" reaction

The triphosphate analogues of AMP, ddAMP, PMEoA and PMEA were also investigated for their kinetic properties with respect to the PRPP synthetases (Tables 3a and b). ddATP showed a 3-fold better affinity for *E.coli* PRPP synthetase than ATP. PMEoApp and PMEApp showed a similar (PMEoApp) or 2-fold higher (PMEApp) K_m than ATP. The V_{max} for ddATP was only 3-fold lower, but the V_{max} for the ANPpp derivatives was at least 300- to 4,000-fold lower than the V_{max} for the natural substrate ATP. This resulted in a comparable phosphorylating efficiency of *E.coli* PRPP synthetase for ATP and ddATP, but a substantially lower efficiency for the ANPpp derivatives (≈ 200- to 7,000-fold) (Table 3a). A similar trend, but less pronounced, was observed for the rat liver isoenzymes. Although the K_m values of the

TABLE 3a
Kinetic properties of adenine nucleotide analogues for PRPP synthetase from *E.coli*

Substrate	*E.coli*[a,b]		
	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m
ATP	23	215	6.8
ddATP	6.8	79	12
PMEoApp	18	0.70	0.039
PMEApp	43	0.05	0.001

[a]The *E.coli* enzyme was used at 0.0001 units per assay (100 μl) for ATP and ddATP (reaction time: 10 min), at 0.04 units per assay for PMEApp (reaction time: 4 hrs), and at 0.002 units per assay for PMEoApp (reaction time: 4 hrs).
[b]Data are the mean of 2 independent experiments.

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TABLE 3b
Kinetic properties of adenine nucleotide analogues for PRPP synthetase from rat liver and human erythrocytes[a]

	Rat liver[b]						Human erythrocytes[c]		
	Type I			Type II					
	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m	K_m (μM)	V_{max} (μmole/mg/hr)	V_{max}/K_m
ATP	7.7	975	127	51	2,335	46	6.9	18	2.5
ddATP	10	283	28	61	740	12	5.3	9.6	1.8
PMEoApp	6.3	15	3.2	33	72	2.2	6.5	0.31	0.047
PMEApp	4.6	1.4	0.23	19	3.0	0.16	9.9	0.03	0.003

[a]Data are the mean of at least 2 to 3 independent experiments.
[b]The rat liver enzymes were used at 0.0002, 0.001, 0.005 and 0.0002 units per assay for ATP (reaction time: 10 min), ddATP (reaction time: 10 min), PMEApp (reaction time: 4 hrs) and PMEoApp (reaction time: 4 hrs), respectively.
[c]The human erythrocyte enzyme was used at 0.0012, 0.0025, 0.005 and 0.0025 units/assay for ATP (reaction time: 10 min), ddATP (reaction time: 10 min), PMEApp (reaction time: 4 hrs), and PMEoApp (reaction time: 4 hrs), respectively.

test compounds were markedly lower for rat liver PRPP synthetase type I than type II (≈ 4- to 7-fold), this was compensated by the V_{max} in the other direction, resulting in comparable V_{max}/K_m values (Table 3b). The human erythrocyte enzyme exhibited significantly lower phosphorylating efficiency for the test compounds than the rat liver type I enzyme, mainly due to the lower V_{max} values (Table 3b).

DISCUSSION

Although PRPP synthetase mainly acts in the direction of PRPP synthesis, the reaction is reversible. The ratio between maximal velocities for the forward reaction (PRPP synthesis) and reverse reaction (ATP and ribose-5-phosphate formation from AMP and PRPP) was reported to be 7-fold (7) and 3.8-fold (18) for the human erythrocyte enzyme. In this study, ratios of 2, 8, 31 and 13 were found for the human erythrocyte, rat liver type I and II and *E.coli* enzymes, respectively. Also, the K_m values for the natural substrate differ markedly from one enzyme to the other (K_m for ATP ranged between 7.7 and 51 μM, and for AMP between 122 and 263 μM). Thus, the affinity of the different PRPP synthetases for their natural substrates varied much more for the forward reaction (formation of PRPP from ATP and ribose-5'-phosphate) than for the reverse reaction (formation of ATP from PRPP and AMP). When ddATP/ddAMP and PMEoApp/PMEoA were used as substrates of these PRPP synthetases, the V_{max} for the forward reaction were generally higher than for the reverse reaction. However, for the reaction of PMEApp/PMEA with the four PRPP synthetases studied, the ratios ranged between 0.2 and 1.5. Our kinetic studies revealed that ddATP and ddAMP are excellent substrates of PRPP synthetase derived from at least 3 different sources. These data are in agreement with the findings of Navé *et al.* (15) who reported that ddAMP is a very good substrate of rat liver and *E.coli* enzymes, more selective than related carbocyclic and acyclic nucleoside phosphonates. Clearly, the enzyme does not discriminate between a ribose or 2',3'-dideoxyribose ring in the sugar part of the adenine nucleotide molecule. Interestingly, the PRPP synthetases show a markedly decreased efficiency of phosphorylation for the isosteric phosphonate derivative d4API, pointing to the importance of an intact phosphate moiety at a right distance from the base (adenine) part. Generally, the acyclic nucleoside phosphonate (ANP) derivatives had significantly lower affinities and much lower V_{max} values than the natural substrate AMP. Nevertheless, all the ANP derivatives that were included in our study may act as alternative substrates for PRPP synthetases from different origin. In the forward reaction, V_{max} decreased in the order of ATP > ddATP > PMEoApp > PMEApp for the different enzymes. Such a trend was not observed for

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the K_m values. Although the velocities at which the different PRPP synthetase enzymes convert the ANP derivatives to the ANPpp's are lower by several orders of magnitude than those measured for their natural substrate AMP, PRPP synthetase can be used to prepare the diphosphorylated derivatives of the ANP analogues so as to perform affinity studies with these compounds against their appropriate target enzymes (i.e. HIV reverse transcriptase, cellular DNA polymerase). It is not clear yet whether the ANP derivatives are phosphorylated in the intact cells by PRPP synthetase to a significant extent. Addition of a PRPP synthetase inhibitor to PMEA-treated HIV-1-infected human CEM cells did not decrease the antiviral efficiency of the test compound (data not shown). However, the PRPP synthetase inhibitor was added at subtoxic concentrations, and it is questionable whether PRPP synthetase is inhibited to a marked extent under these experimental conditions.

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