INTERACTION OF PHOSPHATES OF THE ACYCLIC NUCLEOSIDE PHOSPHONATES WITH NUCLEOSIDE DIPHOSPHATE KINASE FROM YEAST AND BOVINE LIVER

Květoslava HORSKÁ, Ivan VOTRUBA[1,*] and Antonín HOLÝ[2]

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic; e-mail: [1] votruba@uochb.cas.cz, [2] holy@uochb.cas.cz

Received July 12, 2005
Accepted December 12, 2005

The ability of monophosphates of selected acyclic nucleoside phosphonates to serve as substrates for the title NDP kinases was studied. Comparison of the kinetic constants (K_M, V_{max}, k_{cat} and k_{cat}/K_M) estimates indicates that the yeast enzyme catalyzes the phosphorylation of purine and pyrimidine acyclic nucleoside phosphate phosphonates of the 9-[2-(phosphonomethoxy)ethyl] and/or 9-[2-(phosphonomethoxy)propyl] series more efficiently than bovine liver NDP kinase. Yeast enzyme preferentially phosphorylates phosphates of the (phosphonomethoxyalkyl)guanines rather than their adenine counterparts; both enzymes phosphorylate *R*-enantiomers of the 9-[2-(phosphonomethoxy)propyl] series more efficiently than the corresponding *S*-enantiomers. Substitution of the aliphatic chain at the position 3 with hydroxymethyl group considerably increases the substrate activity of phosphate of acyclic nucleoside phosphonate. The resulting substrate activity (k_{cat}/K_M ratio) of all acyclic nucleoside phosphonate phosphates studied is three to five orders of magnitude lower than that for natural NDPs.

Keywords: Enzymatic phosphorylation; Phosphates in acyclic nucleoside phosphonates; Acyclic nucleotide analogs; NTP analogues; NDP kinase; PMEAp; (*R*)-PMPAp; Antivirals.

Acyclic nucleoside phosphonates (ANPs) are currently used in antiviral therapy[1]. (*S*)-HPMPC (Vistide™) is approved for the treatment of cytomegalovirus retinitis in AIDS patients, tenofovir ((*R*)-PMPA) is an antiretroviral acyclic nucleoside phosphonate, whose lipophilic prodrug, tenofovir disoproxil fumarate (Viread™) is used for treatment of HIV infection and bis[(pivaloyloxy)methyl] ester of PME derivative of adenine (adefovir dipivoxil, Hepsera™) was recently approved as anti-HBV drug. In addition to their antiviral potency, these nucleotide analogs exhibit also cytostatic, antiparasitic and immunomodulatory activities[1]. It is generally accepted that the target enzymes of ANPs are the viral and/or cellular replicative polymerases[1]. Interaction with the corresponding polymerases is conditioned by two-step intracellular phosphorylation, which results in

Collect. Czech. Chem. Commun. 2006, Vol. 71, No. 1, pp. 35–43

doi:10.1135/cccc20060035

ANP diphosphates (the analogs of nucleoside 5′-triphosphates). It was shown in our previous studies that the first phosphorylation step is catalyzed by the same cellular nucleoside monophosphate kinases as the natural nucleotides[2–4]. The activation of ANPs in their diphosphates, which is catalyzed by nucleoside diphosphate kinases, has been investigated so far solely for HPMP compounds[2,5–7]. The responses of the other types of antiviral ANPs (namely PME, PMP derivatives[1]) have not yet been investigated.

Nucleoside diphosphate kinases (NDPK; EC 2.7.4.6) are ubiquitous enzymes that catalyze reversible transfer of the terminal high-energy phosphate from an NTP (or dNTP) to an NDP (or dNDP) with a broad specificity. The reaction follows a ping-pong bi bi mechanism with a covalent intermediate[8–10].

NDP kinases show remarkable sequence conservation and have identical active-site residues[9]. The crystal structures of the various NDP kinases are also very similar[11]. It is, therefore, likely that all NDP kinases have identical mechanisms[10]. NDP kinases exist in two different quaternary structures. Sequence analysis and the available X-ray structures suggest that all known eukaryotic and archaebacterial NDP kinases are hexamers, even the mitochondrial Nm 23-H4 form; some bacterial enzymes are tetramers[12].

The NDP kinase active site comprises the nucleophilic histidine (His-118 in human NDP kinase B) and the nucleotide-binding site[12,13]. Histidine in the active site is transiently phosphorylated at the N^{δ} position[14]. There is a single binding site per subunit, which accepts both substrates, donor and acceptor of the phosphate group. Its mode of binding differs from other kinases and appears to be unique at present[12]. The NDP kinase active sites are identical and independent within a tetramer or hexamer. They are also structurally identical in different enzymes and almost all the residues involved in the active site are fully invariant going from bacteria to man[12].

In contrast to the nucleoside monophosphate kinases involved in the first step of the ANPs and/or natural substrate phosphorylation pathway, NDP kinase is nonspecific for the nucleobase or for the 2′ position of the sugar. The NDP kinase substrate binding site accepts purine and pyrimidine moieties of both ribo- and deoxyribonucleoside diphosphates as substrates[10,15,16]. In addition, a major conclusion of the structural and the biochemical data is that critical interactions for catalysis are not made with the protein groups, but, with the 3′-OH of the pentose and the bound metal ion, that is, within the substrate itself. Therefore 3′-hydroxy group is important for the catalysis[13,14,16,17].

The aim of our work was to investigate the interaction of NDP kinase with a series of phosphates of acyclic nucleoside phosphonates, which rep-

resent a group of biologically active nucleotide analogs, where the sugar moiety is replaced with an aliphatic chain, i.e. PME and PMP derivatives. The study was performed with the yeast (*S. cerevisiae*, a tetrameric enzyme[18]), and bovine liver NDP kinases.

RESULTS

In this study we have investigated the ability of NDP kinase from the yeast and bovine liver to use phosphates of purine and pyrimidine acyclic nucleoside phosphonates (Fig. 1, analogs of NDPs) as substrates, i.e. phosphate acceptors. Kinetic parameters shown in Tables I–IV were measured at 0.8 mM ATP. The estimates of catalytic efficacy of yeast NDP kinase towards

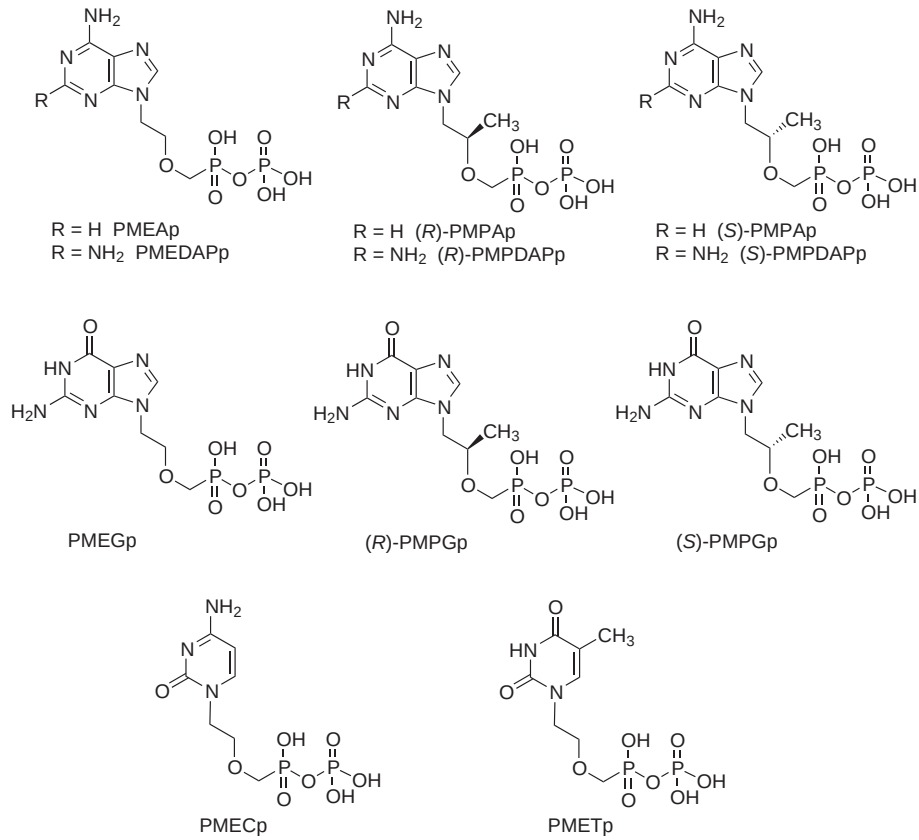

FIG. 1
Structures of phosphates of acyclic nucleoside phosphonates characterized in this study

TABLE I
Substrate activity of 9-[2-(phosphonomethoxy)alkyl]purine phosphates towards NDP kinase from baker's yeast[a]

Substrate	K_M μmol l^{-1}	V_{max} μmol min^{-1} mg^{-1}	k_{cat} s^{-1}	k_{cat}/K_M s^{-1} mol l^{-1}
(S)-HPMPGp	1310 ± 146	52.71 ± 3.11	15.81	12.07×10^3
PMEGp	892 ± 83	4.65 ± 0.16	1.39	1.56×10^3
(*S*)-PMPGp	746 ± 101	1.15 ± 0.04	0.35	0.46×10^3
(*R*)-PMPGp	1500 ± 107	5.18 ± 0.20	1.55	1.04×10^3
PMEDAPp	1600 ± 157	7.86 ± 0.39	2.36	1.47×10^3
PMEAp	1890 ± 175	5.46 ± 0.28	1.64	0.87×10^3
(*S*)-PMPAp	1040 ± 111	1.57 ± 0.07	0.47	0.45×10^3
(*R*)-PMPAp	1060 ± 186	2.97 ± 0.22	0.89	0.84×10^3

[a] Kinetic parameters were measured at 0.8 mM ATP. Data are means ± SEM of three independent experiments.

TABLE II
Substrate activity of 9-[2-(phosphonomethoxy)alkyl]purine phosphates towards NDP kinase from bovine liver[a]

Substrate	K_M μmol l^{-1}	V_{max} μmol min^{-1} mg^{-1}	k_{cat} s^{-1}	k_{cat}/K_M s^{-1} mol l^{-1}
(S)-HPMPGp	958 ± 50	4.15 ± 0.098	1.177	1.23×10^3
PMEGp	1320 ± 131	0.70 ± 0.032	0.199	0.15×10^3
(*S*)-PMPGp	1650 ± 82	0.08 ± 0.002	0.022	0.01×10^3
(*R*)-PMPGp	1290 ± 87	0.29 ± 0.008	0.065	0.05×10^3
PMEDAPp	758 ± 64	0.34 ± 0.011	0.098	0.13×10^3
PMEAp	2090 ± 90	0.40 ± 0.010	0.113	0.05×10^3
(*S*)-PMPAp	1240 ± 131	0.06 ± 0.003	0.017	0.01×10^3
(*R*)-PMPAp	1310 ± 117	0.20 ± 0.009	0.056	0.04×10^3

[a] Kinetic parameters were measured at 0.8 mM ATP. Data are means ± SEM of three independent experiments.

purine ANP phosphates show (Table I) that (*S*)-HPMPGp is the best substrate with k_{cat}/K_M = 12.07 × 10^3 s^{-1} mol l^{-1}. The substrate activities of the other purine ANP phosphates decrease in the order: PMEGp > PMEDAPp > (*R*)-PMPGp > PMEAp > (*R*)-PMPAp > (*S*)-PMPGp > (*S*)-PMPAp. In comparison with natural substrates GDP and ADP (k_{cat}/K_M = 1.81 × 10^6 and/or 0.95 × 10^6 s^{-1} mol l^{-1}, respectively), the phosphorylation efficacy for purine ANP phosphates is lower by three orders of magnitude or more. The k_{cat}/K_M ratio for bovine liver enzyme (Table II; $^{GDP}k_{cat}/K_M$ = 1.09 × 10^6, $^{ADP}k_{cat}/K_M$ = 0.46 × 10^6) is at least a one order of magnitude lower compared with the yeast enzyme (Table I). Also in this case, (*S*)-HPMPGp is the best substrate with k_{cat}/K_M = 1.23 × 10^3 s^{-1} mol l^{-1} and the efficacy of phosphorylation of other purine ANP phosphates decreases in the order: PMEGp > PMEDAPp > PMEAp > (*R*)-PMPGp > (*R*)-PMPAp > (*S*)-PMPAp > (*S*)-PMPGp. This pattern differs from the previous one, which shows a significant preference to guanine ANPp over adenine derivatives. In both the cases, *R*-enantiomers of guanine and adenine PMP derivatives are better substrates than their

TABLE III
Substrate activity of 1-[2-(phosphonomethoxy)ethyl]pyrimidine phosphates towards NDP kinase from baker's yeast[a]

Substrate	K_M µmol l^{-1}	V_{max} µmol min^{-1} mg^{-1}	k_{cat} s^{-1}	k_{cat}/K_M s^{-1} mol l^{-1}
PMECp	2030 ± 187	1.77 ± 0.08	0.53	2.62 × 10^2
PMETp	1500 ± 144	1.39 ± 0.05	0.42	2.78 × 10^2

[a] Kinetic parameters were measured at 0.8 mM ATP. Data are means ± SEM of three independent experiments.

TABLE IV
Substrate activity of 1-[2-(phosphonomethoxy)ethyl]pyrimidine phosphates towards NDP kinase from bovine liver[a]

Substrate	K_M µmol l^{-1}	V_{max} µmol min^{-1} mg^{-1}	k_{cat} s^{-1}	k_{cat}/K_M s^{-1} mol l^{-1}
PMECp	2040 ± 300	0.19 ± 0.015	0.054	0.27 × 10^2
PMETp	1830 ± 241	0.09 ± 0.007	0.026	0.14 × 10^2

[a] Kinetic parameters were measured at 0.8 mM ATP. Data are means ± SEM of three independent experiments.

S-counterparts. Interestingly, the K_M values of studied (*S*)-HPMPGp, (*R*)-PMPGp, (*S*)-PMPGp and PMEDAPp, which approximately reflect an inverse measure of the binding affinity between the enzyme and its substrate, also significantly differ for both enzymes. A comparison of the kinetic constants (K_M, V_{max}, k_{cat} and k_{cat}/K_M) estimates indicates that the yeast enzyme catalyzes phosphorylation of purine ANP phosphates more efficiently than the NDP kinase from bovine liver.

Pyrimidine ANP phosphates, PMECp and PMETp, are also better substrates for yeast enzyme than for bovine NDP kinase (Tables III and IV). A comparison of their K_M values shows nearly the same binding affinity towards the both enzymes. In the bovine liver catalyzed reaction, PMECp possesses higher substrate activity than PMETp, while the substrate activity of the both pyrimidine derivatives in the yeast catalyzed reaction is the same (Tables III and IV). PMEUp does not serve as a substrate either for yeast or bovine liver enzyme (data not shown). The k_{cat}/K_M values for purine ANP phosphates are mostly higher than those for pyrimidine derivatives (Tables I–IV).

DISCUSSION

Nucleotide analogs are currently used in antiviral therapy[1]. However, only the triphosphate analogs act as substrates of the viral replicases. It is generally accepted that the antiviral analogs are phosphorylated by the same cellular kinases as the natural nucleotides[13,17]. The activation of nucleosides into their mono- and diphosphate forms is catalyzed by nucleoside kinases and nucleoside monophosphate kinases with various degrees of specificity[2].

The data presented in this comparative study show a considerably different phosphorylation pattern of both the purine and pyrimidine ANP phosphates catalyzed by yeast and/or bovine liver NDP kinases of commercial origin. The yeast NDP kinase has at least one order of magnitude higher catalytic efficacy towards purine and pyrimidine ANP phosphates than the bovine liver enzyme. Moreover, our data on kinetic parameters estimates show that both enzymes differ also with respect of the guanine derivatives recognition. In the PMP series, bovine liver enzyme preferentially phosphorylate *R*-enantiomers, which perfectly correlates with their biological activity[1]. Substrate activity of phosphates of PMP and PME series was compared with (*S*)-HPMPGp because the compound containes the CH_2OH group at the position 3 of its chain. As was shown in our previous study[4], the presence of OH mimics 3′-hydroxy group of the ribose moiety of a natural nucleotide; hence, both enzymes phosphorylate (*S*)-HPMPGp with a highest

efficiency. Replacement of hydrogen in PME derivatives by CH_3 group at the position 3 of the aliphatic chain (PMP derivatives) considerably decreases the substrate activity of these nucleotide analogs towards NDP kinase. These findings are consistent with the previous observation that all modifications of the phosphate chain and ribose moiety lead to a large decrease in the catalytic efficacy of NDP kinase, as is reflected by considerably low values of k_{cat}/K_M (refs[10,15]). It is highly probable that the aliphatic chain of studied PME and PMP derivatives does not permit an appropriate interaction with Asn-115 and Lys-12 in the enzyme active site via hydrogen bonds[10].

Surprisingly, replacement of natural adenine with 2,6-diaminopurine in the PME series enhanced significantly the catalytic efficacy of the enzyme.

Our study shows that NDP kinase can be considered an enzyme candidate catalyzing the phosphate transfer in the last intracellular activating step of the acyclic nucleoside phosphonates thus mediating their antiviral and/or cytostatic activity. The phosphorylation of ANP phosphates with other enzymes[19], which are able to convert NDP to NTP, i.e. 3-phosphoglycerate kinase, creatine kinase or pyruvate kinase, remains to be examined.

EXPERIMENTAL

Chemicals

Nucleotides, NDP kinase from bovine liver, NDP kinase from baker's yeast and other reagents were obtained from Sigma. ANP phosphates were synthesized by the modified morpholidate method as was described by Hájek et al.[20] All other chemicals and materials were commercial products of Sigma, such as ATP, tetrabutylammonium hydrogensulfate and corresponding buffers and salts.

Kinetic Experiments

Standard reaction mixtures contained 100 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.8 mM ATP and an appropriate amount of substrate (150–4500 μmol l^{-1}, 12 concentrations for each substrate) as well as of enzyme (4–100 mU). The reaction mixtures were incubated at 30 °C for 10 min (NDP kinase from baker's yeast) or for 30 min (NDP kinase from bovine liver). The reactions were stopped by adding the same volume of 10% TCA (5 min, 0 °C). TCA was removed by shaking for 10 min with the same volume of a mixture tri-*n*-octylamine/1,1,2-trichloro-1,2,2-trifluoroethane (1:5). After centrifugation (11 000 *g*, 10 min) the aliquots of the upper layer were analyzed by HPLC chromatography in a Waters Aliance 2795 system (2996 PDA Detector, PDA Software Millenium[32]) equipped with 15 cm × 4 mm Supelcosil™ LC 18T 3 μm reverse-phase column. The non-linear gradient (mixed at curves 4 and 8) at a flow rate of 0.75 ml min^{-1} was used: solvent A, 50 mM potassium dihydrogenphosphate, 3 mM tetrabutylammonium hydrogensulfate; solvent B, 50 mM potassium dihydrogenphosphate, 3 mM tetrabutylammonium hydrogensulfate with 30% ace-

tonitrile; pH 3.1 for compounds (*S*)-HPMPAp, (*S*)-HPMPGp, (*R*)-PMPAp, (*S*)-PMPAp, PMETp, pH 5.1 for PMECp and pH 6.1 for (*S*)-PMPGp, (*R*)-PMPGp, PMEDAP, PMEAp, PMEGp.

Kinetic constants (K_M and V_{max}) were determined from the Lineweaver–Burk plots. Data based on results from at least three independent experiments were evaluated by the non-linear regression method (BioSoft EnzFitter, 32 bit version for Windows). For k_{cat} calculation of baker's yeast NDP kinase[18] and NDP kinase from bovine liver[21] the corresponding subunit molecular weight was used.

Abbreviations

(*S*)-HPMPGp, (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine phosphate; PMEGp, 9-[2-(phosphonomethoxy)ethyl]guanine phosphate; (*S*)-PMPGp, (*S*)-9-[2-(phosphonomethoxy)propyl]guanine phosphate; (*R*)-PMPGp, (*R*)-9-[2-(phosphonomethoxy)propyl]-guanine phosphate; PMEDAPp, 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine phosphate; PMEAp, 9-[2-(phosphonomethoxy)ethyl]adenine phosphate; (*S*)-PMPAp, (*S*)-9-[2-(phosphonomethoxy)propyl]adenine phosphate; (*R*)-PMPAp, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine phosphate; PMETp, 1-[2-(phosphonomethoxy)ethyl]thymine phosphate; PMECp, 1-[2-(phosphonomethoxy)ethyl]cytosine phosphate; PMEUp, 1-[2-(phosphonomethoxy)ethyl]uracil phosphate; PME, 9-[2-(phosphonomethoxy)ethyl]; PMP, 9-[2-(phosphonomethoxy)propyl]; DTT, dithiothreitol; TCA, trichloroacetic acid.

Supported by the Program of targeted projects of the Academy of Sciences of the Czech Republic No. S4055109 under research project Z4055905.

REFERENCES

1. Holý A.: *Curr. Pharm. Des.* **2003**, *9*, 2567.
2. Merta A., Votruba I., Jindřich J., Holý A., Cihlář T., Rosenberg I., Otmar M., Hervé T. Y.: *Biochem. Pharmacol.* **1992**, *44*, 2067.
3. Krejčová R., Horská K., Votruba I., Holý A.: *Collect. Czech. Chem. Commun.* **2000**, *65*, 1653.
4. Krejčová R., Horská K., Votruba I., Holý A.: *Biochem. Pharmacol.* **2000**, *60*, 1907.
5. Cihlář T., Votruba I., Horská K., Liboska R., Rosenberg I., Holý A.: *Collect. Czech. Chem. Commun.* **1992**, *57*, 661.
6. Cihlář T., Chen M. S.: *Mol. Pharmacol.* **1996**, *50*, 1502.
7. Terry B. J., Mazina K. J., Tuomari A. V., Haffey M., Hagen M., Feldman A., Slusarchyk W. A., Young M. G., Zahler R., Field A. K.: *Antiviral Res.* **1988**, *10*, 235.
8. Sheu K. F., Richard J. P., Frey P. A.: *Biochemistry* **1979**, *18*, 5548.
9. Krejčová R., Horská K.: *Chem. Listy* **1997**, *91*, 466.
10. Lascu I., Gonin P.: *J. Bioenerg. Biomembr.* **2000**, *32*, 237.
11. Lascu I., Giartosio A., Ransac S., Erent M.: *J. Bioenerg. Biomembr.* **2000**, *32*, 227.
12. Janin J., Dumas Ch., Moréra S., Xu Y., Meyer P., Chiadmi M., Cherfils J.: *J. Bioenerg. Biomembr.* **2000**, *32*, 215.
13. Gallois-Montbrun S., Véron M., Deville-Bonne D.: *Min. Rev. Med. Chem.* **2004**, *4*, 361.
14. Gonin P., Xu Y. W., Milon L., Dabernat S., Morr M., Kumar R., Lacombe M.-L., Janin J., Lascu I.: *Biochemistry* **1999**, *38*, 7265.

15. Schneider B., Sarfati R., Deville-Bonne D., Véron M.: *J. Bioenerg. Biomembr.* **2000**, *32*, 317.
16. Schaertl S., Konrad M., Geeves M. A.: *J. Biol. Chem.* **1998**, *273*, 5662.
17. Bourdais J., Biondi R., Sarfati S., Guerreiro C., Lascu I., Janin J., Véron M.: *J. Biol. Chem.* **1996**, *271*, 7887.
18. Jong A. Y., Ma J. J.: *Arch. Biochem. Biophys.* **1991**, *291*, 241.
19. Feng J. Y., Parker W. B., Krajewski M. L., Deville-Bonne D., Veron M., Krishnan P., Cheng Y.-C., Borroto-Esoda K.: *Biochem. Pharmacol.* **2004**, *68*, 1879.
20. Hájek M., Matulová N., Votruba I., Holý A., Tloušťová E.: *Biochem. Pharmacol.* **2005**, *70*, 894.
21. Abdulaev N. G., Karaschuk G. N., Ladner J. E., Kakuev D. L., Yakhyaev M. T., Tordova M., Gaidarov I. O., Popov V. I., Fujiwara J. H., Chinchilla D., Eisenstein E., Gilliland G. L., Ridge K. D.: *Biochemistry* **1998**, *37*, 13958.