PHOSPHORYLATION OF PURINE (PHOSPHONOMETHOXY)ALKYL DERIVATIVES BY MITOCHONDRIAL AMP KINASE (AK2 TYPE) FROM L1210 CELLS

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Substrate activity of purine (phosphonomethoxy)alkyl derivatives towards mitochondrial AMP kinase (AK2 type) from L1210 cells was studied. The native AMP kinase, purified nearly to homogeneity, is a monomer with molecular weight 26 kDa. The purified AMP kinase is specific for natural adenine nucleotides (AMP and dAMP) as phosphate acceptors but has a broad specificity to nucleoside 5′-triphosphates as phosphate donors. In addition to adenine acyclic nucleotide analogues, the enzyme is capable of phosphorylating also analogous derivatives containing 2,6-diaminopurine moiety. Kinetic data show that the substrate activity of these acyclic nucleoside phosphonates towards AK2 isoenzyme decreases in the order (*S*)-HPMPA > (*R*)-PMPA > PMEA > PMEDAP > (*S*)-PMPDAP > (*R*)-PMPDAP >> (*S*)-PMPA. Acyclic nucleotide analogues do not exhibit any inhibitory activity towards AK2 isoenzyme.

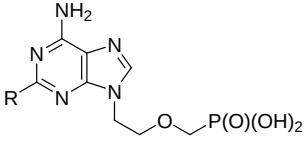
Key words: Enzymatic phosphorylation; Acyclic nucleotide analogues; Nucleotides; Phosphonates; Phosphates; AMP kinase; AK2; L1210 cells; PMEA; PMPA; PMEDAP; Antivirals.

Acyclic nucleoside phosphonate analogues (ANP) of both purine and pyrimidine bases exhibit extensive antiviral activity and numerous other biological effects. They contain the catabolically stable P–C bond in the phosphonomethoxy group which simulates the phosphate group. Several sub-classes, each showing a different antiviral activity spectrum, can be distinguished: the 3-hydroxy-2-(phosphonomethoxy)propyl (HPMP) derivatives, the 2-(phosphonomethoxy)ethyl (PME) derivatives, the 3-fluoro-2-(phosphonomethoxy)propyl (FPMP) derivatives and the 2-(phosphonomethoxy)propyl (PMP) derivatives[1].

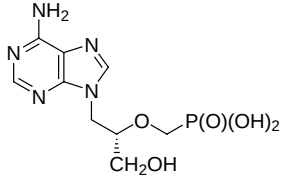
The most extensively studied analogue in the group of adenine derivatives is PMEA (adefovir) which exhibits potent activity against herpesviruses, human hepatitis B virus and retroviral (human immunodeficiency virus type 1 and 2, simian immunodeficiency virus, visna virus and feline immunodeficiency virus) infections[2]. Moreover, its significant cytostatic

potency against various neoplasias[3,4] and immuno-modulatory effects[5] have been also reported. Adefovir dipivoxil ([9-(2-({bis-[(pivaloyloxy)methoxy] phosphoryl}methoxy)ethyl]adenine, bis-POM PMEA), an orally bioavailable prodrug of adefovir, is currently being evaluated for treatment of hepatitis B virus infections[2,6]. PMEA and/or PMEDAP (2,6-diaminopurine derivative) treatment of SD rat lymphomas significantly prolonged the mean survival time of tumor-bearing animals[7] and induced apoptosis[8,9]. Hatse *et al.*[10] found a strong differentiation-inducing effect of PMEA on rat choriocarcinoma cells. PMEA and its oral prodrug [bis(POM)-PMEA] have powerful antiarthritic effect: in a model of adjuvant-induced arthritis in Lewis rats both compounds inhibit arthritic paw swelling, splenomegaly and fibroadhesive perisplenitis[11].

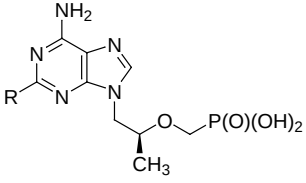
(*S*)-HPMPA inhibits replication of many DNA viruses[12]. This compound and its cyclic phosphonate derivative possess also antitrypanosomal, antileishmanial and antimalarial activity *in vitro* and *in vivo*[13,14].



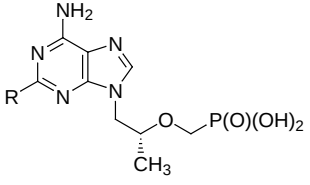
R = H, PMEA
R = NH_2, PMEDAP



(*S*)-HPMPA



R = H, (*S*)-PMPA
R = NH_2, (*S*)-PMPDAP



R = H, (*R*)-PMPA
R = NH_2, (*R*)-PMPDAP

(*R*)-PMPA (tenofovir) proved selective inhibitory effect on human immunodeficiency virus types 1 and 2, feline immunodeficiency virus and Moloney murine sarcoma virus (MSV) in cell cultures; its selectivity is higher than that of PMEA (ref.[15]). Tenofovir has demonstrated potent anti-SIV activity in rhesus macaques[2,16,17]. Tenofovir disoproxil [bis(POC)PMPA] (ref.[18]), an oral prodrug of tenofovir, is in Phase III clinical evaluation as antiretroviral agent. (*R*)- and (*S*)-enantiomers of PMPA also

greatly enhance the secretion of tumor necrosis factor-alpha and interleukin-10, factors known to play a role in HIV virus replication[19].

Once inside the cells, the ANP compounds need to be activated to their diphosphoryl derivatives (ANPpp) by cellular enzymes[1,20]. The antiviral and cytostatic effects of ANPs result from selective interactions of their diphosphates with the viral and/or cellular replicative DNA polymerases[21,22]. On the basis of the structural resemblance to natural 2′-deoxynucleoside 5′-triphosphates, these diphosphates act as substrate/inhibitors during the DNA polymerase reaction. In contrast to PMEApp or PMPApp, which are DNA-chain terminators, (*S*)-HPMPApp contains the hydroxy group which permits certain DNA-chain elongation *de novo* associated with the incorporation of several analogue molecules[23,24].

The nucleoside monophosphate kinases have been shown to catalyze the first step of the ANP phosphorylation (*i.e.* to ANPp) in adenine[25], guanine[26] and cytosine[27,28] series. This process is enantiospecific, *i.e.* the absolute configuration at the side chain is important for this reaction[25–28].

We were particularly interested in the phosphorylation of the acyclic nucleoside phosphonates derived from 2,6-diaminopurine which exhibit potent biological activity[29]; they undergo massive phosphorylation in the cells but the enzymes responsible for this activation were not yet identified. In our recent papers, we have demonstrated that isoenzymes of GMP kinase from L1210 cells[30] do not accept the 2,6-diaminopurine derivatives as substrates[26]. It is evident that another phosphorylating system must be responsible for their phosphorylation in cell.

AMP(dAMP) kinase (EC 2.7.4.3, AK) is a key enzyme in the activation of adenine derivatives PMEA and (*S*)-HPMPA (refs[25,31]). In vertebrates, three isoenzymes, AK1, AK2 and AK3 have been identified. AK1 is present mainly in the cytosol of skeletal muscle, brain and erythrocytes. AK2 is located in the mitochondrial membrane, while the isoenzyme AK3 which uses GTP as a phosphate donor is localized in the mitochondrial matrix[32].

This communication is aimed at identification of the AMP kinase isoenzyme from L1210 cells responsible for the phosphorylation of ANPs containing adenine and 2,6-diaminopurine and at detailed study of their function as substrates of AMP kinase. This enzyme preparation was used for investigation of its interaction with (*R*)- and (*S*)-PMPA as well as with structurally related highly biologically active 2,6-diaminopurine derivatives PMEDAP, (*R*)-PMPDAP and (*S*)-PMPDAP. For comparison, we also included in this group PMEA and (*S*)-HPMPA which have been extensively studied in the past[20,25]. AK2 isoenzyme of AMP kinase was postulated to be their phosphorylating enzyme in the crude extracts of human lymphoid cells[31].

EXPERIMENTAL

Materials

[U-^{14}C]-AMP (21.8 GBq mmol^{-1}) was from NEN (Belgium). [^{3}H]-PMEA (630 GBq mmol^{-1}), [^{3}H]-PMEDAP (470 GBq mmol^{-1}), [^{3}H]-(*S*)-HPMPA (460 GBq mmol^{-1}), [^{3}H]-(*R*)-PMPA (430 GBq mmol^{-1}), [^{3}H]-(*S*)-PMPA (510 GBq mmol^{-1}), [^{3}H]-(*R*)-PMPDAP (27 GBq mmol^{-1}), [^{3}H]-(*S*)-PMPDAP (570 GBq mmol^{-1}) were synthesized in the Central Laboratory of Radioisotopes, Charles University (Prague, Czech Republic). All nonlabelled acyclic nucleoside phosphonates were synthesized as described by Holý *et al.*[33–35]. Proteinase inhibitors (aprotinin, bestatin, leupeptin, pepstatin), 2-sulfanylethan-1-ol, PMSF, BSA, DTNB (2,2′-dinitro-5,5′-disulfanediyldibenzoic acid), Ap_5A [P^1,P^5-di(adenosine-5′) pentaphosphate], NEM (*N*-ethylmaleimide), MOPS (3-morpholinopropane-1-sulfonic acid), creatine phosphate, creatine kinase as well as chicken muscle myokinase were products of Sigma. Polygram® Cel 300 PEI/UV_{254} for TLC Chromatography was from Macherey–Nagel (Germany). Polybuffer exchanger PBE 94, polybuffers PB 74 and PB 96 for chromatofocusing, columns PD-10 were purchased from Pharmacia P-L Biochemicals (Sweden). DEAE cellulose DE-52 was from Whatman (Great Britain). Centriprep-10 and Centricon-10 were from Amicon (U.S.A.).

Cells

Mouse leukemia L1210 cells were grown in inbred DBA/2 male mice (25 g) after i.p. transfer of 10^5 cells and harvested eight days after inoculation. The cells were released from the peritoneum in phosphate-buffered saline (PBS), washed twice with 10 mM potassium phosphate buffer with 0.15 M KCl and 5 mM 2-sulfanylethan-1-ol and stored at –70 °C.

Adenylate Kinase Assay

During the purification procedure, the reaction mixtures for estimation of AMP kinase activity contained in final volume of 20 μl: 40 mM HEPES (Na^+), pH 8.0, 15 mM $MgCl_2$, 3 mM ATP, 2 mM 2-sulfanylethan-1-ol, 0.1 mM [U-^{14}C]-AMP (0.74 GBq mmol^{-1}) and 2 μl of tested fractions. The reactions were carried out at 37 °C for 5 min and stopped by spotting 5 μl aliquot onto a poly(ethyleneimine)cellulose TLC plate (pretreated by soaking in distilled water and dried) which had been prespotted with 0.01 μmol of each substrate and product as carriers. The plate was developed in the solvent system 0.8 M LiCl in 0.8 M CH_3COOH. The product spots were visualized under UV light (254 nm), cut out and evaluated for radioactivity in a toluene-based scintillation fluid. One enzyme unit (U) is defined as the amount of enzyme that catalyzes phosphorylation of 1 μmol of AMP per minute under the conditions of the standard assay. Specific activity of an enzyme is expressed in enzyme units per milligram of protein.

Kinetic Experiments

Kinetic constants (K_m and V_{max}) were determined from the Lineweaver–Burk plots using varying concentration of AMP (2–100 μM), PMEDAP, (*S*)-PMPDAP (100–1 300 μM), (*R*)-PMPA, PMEA (200–2 000 μM), (*S*)-HPMPA and (*R*)-PMPDAP (200–3 000 μM). For kinetic assays with AMP as a substrate, the reaction mixture (20 μl) contained 0.5 mM ATP, 40 mM HEPES (Na^+), pH 8.0, 1.5 mM $MgCl_2$, 2 mM 2-sulfanylethan-1-ol, 0.4 mg ml^{-1} BSA and 10.3 μU AMP kinase

from L1210 cells or 20 μU chicken muscle myokinase. The reaction mixtures were incubated for 5–15 min at 37 °C. The kinetic assays with the ANPs as substrates were performed in the reaction mixture (20 μl) containing 0.2 mM ATP, 40 mM HEPES (Na^+), pH 8.0, 1 mM $MgCl_2$, 2 mM 2-sulfanylethan-1-ol, 0.4 mg ml^{-1} BSA, 5 mM creatine phosphate, 0.05 mg ml^{-1} creatine kinase and an appropriate amount of AMP kinase, which was approximately 500–1 000 times higher than that used for phosphorylation of AMP. The reaction mixtures were incubated for 90–180 min at 37 °C.

Data (Lineweaver–Burk plots) based on results from four independent experiments were evaluated by the nonlinear regression method (Biosoft EnzFitter, 32 bit version for Windows).

Denaturing Gel Electrophoresis of Proteins

SDS-PAGE system according to Laemmli[36] (15% polyacrylamide) was used for the analysis of the purified AMP kinase isoenzyme. Samples were heated at 95 °C for 10 min (in some cases after concentration by vacuum evaporation) in 30 mM Tris-HCl, pH 6.8, 5% SDS, 5% glycerol (v/v) and 5% 2-sulfanylethan-1-ol. LMW calibration kit was used as a protein standard. The gels were stained with silver (Ag^+) (ref.[37]).

Native Gel Electrophoresis of Proteins

Gradient slab gels (4–30%) were run to equilibration (2 500 V h) on a 2050 MIDGET electrophoresis unit (LKB). The gels had been prerun in TBE buffer for 30 min, pH 8.3, containing 9 mM 2-sulfanylethan-1-ol; LMW and HMW calibrations kits were used as protein standards. One part of the gel was stained with silver (Ag^+) (ref.[37]) and the other was cut into 2 mm slices. AMP phosphorylating activity was determined after extraction of each slice with an assay mixture which contained (150 μl): 40 mM HEPES (Na^+), pH 8.0, 15 mM $MgCl_2$, 2 mM 2-sulfanylethan-1-ol, 3 mM ATP and 30 μM [U-^{14}C]-AMP (2.47 GBq $mmol^{-1}$).

Buffers

Buffer A: 10 mM Tris-HCl, pH 7.4, 2 mM 2-sulfanylethan-1-ol, 1 mM PMSF, aprotinin (2 μg ml^{-1}), bestatin (40 μg ml^{-1}), leupeptin (2 μg ml^{-1}), pepstatin A (4 μg ml^{-1}).
Buffer B: 50 mM Tris-HCl, pH 8.0, 2 mM 2-sulfanylethan-1-ol, 1 mM PMSF.
Buffer C: 5 mM Tris-HCl, pH 7.5, 2 mM 2-sulfanylethan-1-ol, 1 mM PMSF.
Buffer D: 25 mM Tris-acetate, pH 8.8.
Buffer E: Polybuffer 74–Polybuffer 96 (1 : 1) adjusted with CH_3COOH to pH 5.0.
Buffer TBE: 90 mM Tris-boric acid, pH 8.3, 2 mM EDTA.

Purification of Mitochondrial Adenylate Kinase

AMP kinase was purified from the crude cell extract by chromatography on DEAE cellulose and hydroxyapatite columns and finally by chromatofocusing. All procedures were performed at 4 °C.

Crude cell extract. The frozen L1210 cells (36 g) were thawed and suspended in 72 ml of buffer A extracted by freeze-thawing (three times) and homogenized in Dounce tissue grinder (Wheaton, 15 strokes of pestle B). The extract was centrifuged at 30 000 *g* for 25 min and then at 100 000 *g* for 100 min. Nucleic acids were precipitated with streptomycin sulfate

(0.7%) and removed by centrifugation at 20 000 *g* for 15 min. The supernatant was used for next purification steps.

Ammonium sulfate cut (0–80%). Proteins were concentrated by salting out in 80% ammonium sulfate. The precipitate was left to stand overnight at 4 °C and stored at –20 °C (fraction I).

Chromatography on DEAE cellulose. An aliquot of fraction I (one sixth) was dissolved in 5 ml of buffer B, centrifuged at 20 000 *g* for 30 min and the supernatant was desalted on PD-10 columns equilibrated in buffer B. Final volume 7 ml (84.33 mg of protein) was applied onto a DEAE cellulose column (1.3 × 6.4 cm) in buffer B. Proteins having AMP kinase activity were eluted with five column volumes of buffer B (0.4 ml min^{-1}, fraction volume 4 ml). Active fractions were pooled, precipitated with solid ammonium sulfate to 80% saturation and stored at –20 °C (fraction II).

Chromatography on hydroxyapatite. Fraction II was dissolved in 5 ml of buffer C and centrifuged at 20 000 *g* for 30 min. The supernatant was desalted on PD-10 columns and the eluate (final volume 7 ml, 16.44 mg protein) was applied onto a hydroxyapatite column (1.2 × 6.5 cm) equilibrated with buffer C. After washing the column with one column volume of buffer C (0.2 ml min^{-1}, fraction volume 4 ml), the enzyme activity was eluted stepwise with one column volume each of buffer C containing 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8 and 10% ammonium sulfate. The fractions with the enzyme activity were pooled, concentrated using Centriprep-10 (2 000 *g*, 90 min) and desalted on PD-10 columns pretreated with buffer D (fraction III).

Chromatofocusing. The polybuffer exchanger PBE 94 column (0.8 × 40 cm) was equilibrated with buffer D and washed with 6 ml of buffer E. Fraction III (5 ml, 9.56 mg of protein) was then applied onto the column and focused at the internal pH gradient created by elution with the same buffer E (0.2 ml min^{-1}, 3 ml fractions). Each of the two resulting peaks (A_1, A_2) with AMP kinase activity was pooled and concentrated (Centricon-10, 4 000 *g*, 60 min). The purified enzymes were stabilized with BSA (final concentration, 0.4 mg ml^{-1}) and stored at –70 °C. The concentration of proteins was determined by the method of Bradford[38] with bovine serum albumin as the standard.

Identification of AMP Kinase Isoenzyme

Isoenzymes AK1, AK2 and AK3 exhibit different behaviour in the presence of Ap_5A (refs[39,40]), DTNB (ref.[41]) and NEM (ref.[42]). In all the experiments mentioned in this paragraph, the amount of protein A_1, protein A_2 and myokinase from chicken muscle were 7.7, 7.6 and 20 μU, respectively. The concentration of [U-^{14}C]-AMP was 20 μM for A_1 and A_2 and 50 μM for myokinase from chicken muscle, as well.

Ap_5A – Reaction mixtures contained 40 mM HEPES (Na^+), pH 8.0, 1.5 mM $MgCl_2$, 2 mM 2-sulfanylethan-1-ol, 0.5 mM ATP and Ap_5A (seven concentrations in the range of 0–300 μM). The incubation time was 5, 15 and 30 min at 37 °C.

DTNB – Reaction mixtures contained 40 mM HEPES (Na^+), pH 7.0, 1.5 mM $MgCl_2$, 0.5 mM ATP and DTNB (six concentrations in the range of 0–2.5 mM) and were preincubated without substrate at 37 °C for 15 min. The reaction was started after the substrate addition and the incubation continued at 37 °C for 5, 15 and 30 min.

NEM treatment (1 mmol l^{-1}) of the enzyme was carried out in 20 mM sodium MOPS, pH 8.0 and 0.2 M NaCl at 25 °C for 1 h before the reaction assay started. The reaction then con-

tinued in the presence of 40 mM HEPES (Na^+), pH 8.0, 1.5 mM $MgCl_2$ and 0.5 mM ATP at 37 °C for 10 min.

RESULTS

Adenylate kinase catalyzing the phosphorylation of AMP to ADP from mouse leukemic L1210 cells has been purified nearly to homogeneity. The isolation procedure consisted of four steps (Table I). For separation of AMP and NDP kinase present in the crude extract, we attempted to use chromatography on DEAE-cellulose, phosphocellulose, AMP-Sepharose, Sephacryl 300, hydroxyapatite and Blue Sepharose. Only the hydroxyapatite column was capable of separating AMP and NDP kinase activities. The active fractions (at the ammonium sulfate concentration 1.8–4.3%) had to be immediately concentrated using Centriprep-10, frozen in liquid N_2, stored at –70 °C and later on directly used for chromatofocusing. We have found that AMP kinase from L1210 cells exists in two forms denoted A_1 (pI 7.2) and A_2 (pI 6.9) (Fig. 1), respectively. Specific activity substantially increased (40-fold) in comparison with the 100 000 *g* supernatant (Table I). The efficiency of the purification procedure is shown in Fig. 2.

The proteins A_1 and A_2 were free of NDP kinase activity and exhibited a single polypeptide band on silver-stained sodium dodecyl sulfate polyacrylamide gel with the molecular weight 26 kDa in accordance with published

TABLE I
Purification of AMP kinase from L1210 cells[a]

Purification step	Total protein mg	Enzyme activity mU	Specific activity mU mg^{-1}	Fold purification
100 000 g Supernatant	123.3	6 893	52	1
0–80% SA	84.33	6 276	74	1.44
DEAE Cellulose	16.44	5 463	332	6.43
Hydroxyapatite	9.56	4 485	469	9.07
A_1[b]	1.22	2 387	1 960	37.9
A_2[b]	0.91	1 886	2 070	40.04

[a] Estimated from one purification run (6 g of frozen cells). [b] Products of chromatofocusing.

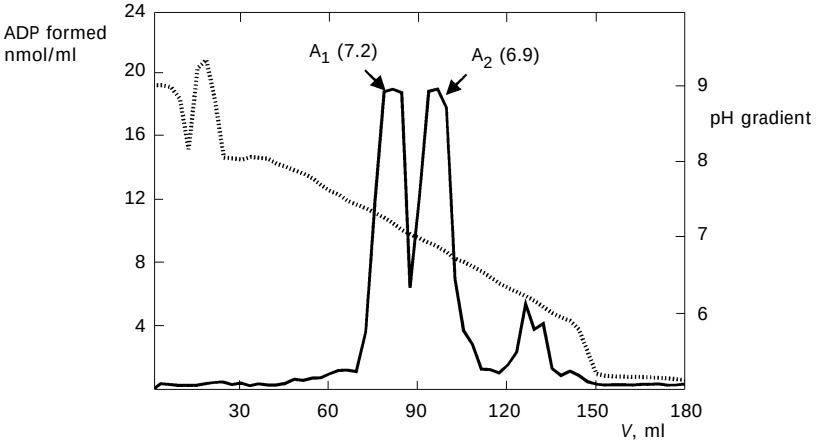


FIG. 1
Chromatofocusing of prepurified AMP kinase

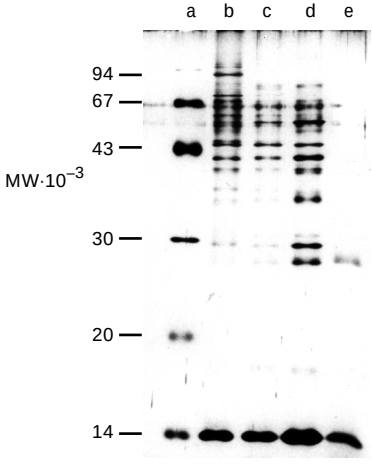


FIG. 2
Purification of mitochondrial AMP kinase – SDS-PAGE pattern. Lanes (from left to right): a LMW calibration kit (phosphorylase b 94 kDa, BSA 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20 kDa, α-lactalbumin 14 kDa); b 0–80% ammonium sulfate cut; c active fractions from DEAE cellulose; d active fractions from hydroxyapatite; e AMP kinase (A_1) after chromatofocusing

data (AK2 from bovine heart) (ref.[43]). After electrophoresis on gradient polyacrylamide gel under nondenaturing conditions (in the presence of 2-sulfanylethan-1-ol), the AMP kinase was found to migrate both as trimer and oligomers (Fig. 3).

We have found that both forms of AMP kinase from L1210 cells are specific for adenine nucleotides (AMP and dAMP) as phosphate acceptors; non-adenine nucleotides (GMP, CMP and UMP) are not tolerated (data not shown). On the other hand, the binding site of the enzyme for the phosphate-donating nucleoside 5′-triphosphate is much less specific in its base requirements (Table II). The ability of nucleoside 5′-triphosphates to serve as phosphate donor for both proteins A_1 and A_2 catalyzing ADP formation from AMP decreases in the order ATP = dATP > UTP > CTP > GTP > dCTP > dGTP > dTTP. Surprisingly, under the experimental conditions used, CTP and UTP could replace ATP with the relatively high efficiency (about 30% for CTP and even 60% for UTP). Similar results were described for AMP kinase from *Sulfolobus acidocaldarius*[44]. On the other hand, chicken muscle myokinase (Table II) prefers ATP and dATP for the phosphotransferase reaction and its activity with pyrimidine nucleoside 5′-triphosphates is – except for dCTP – mostly marginal.

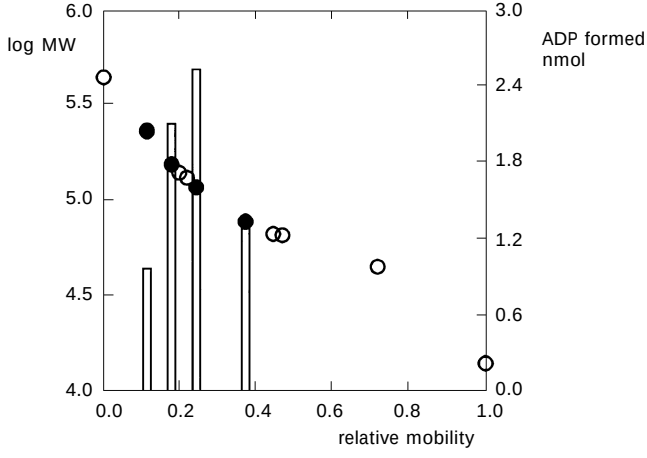


FIG. 3
Gradient PAGE pattern of AMP kinase (A_1). (○) Molecular weight standards (from left to right: ferritin, catalase, lactate dehydrogenase, albumin dimer, albumin 67 kDa, albumin 66 kDa, ovalbumin, α-lactalbumin); (●) AMP kinase (A_1) oligomers (from right to left: trimer, pentamer, hexamer, nonamer (overlaped with catalase))

AMP kinase type AK1 – represented in our experiments by chicken muscle myokinase – is highly susceptible to the inactivation by thiol reagents (DTNB and NEM) as well as by Ap_5A. On the basis of the characteristic behaviour of isoenzymes AK1 and AK2 in the presence of these inhibitors, we were able to identify the type of AMP kinase present in L1210 cells which is responsible for the phosphorylation of adenine acyclic nucleoside phosphonate analogues: both proteins A_1 and A_2 are mitochondrial AMP kinase type AK2 (Table III). Kinetic properties of both proteins A_1 and A_2 are nearly the same, *i.e.* ${}^{A1}K_m$ = 18.7 μmol l^{-1}, ${}^{A1}V_{max}/{}^{A1}K_m$ = 0.197 l min^{-1} mg^{-1} and ${}^{A2}K_m$ = 20.5 μmol l^{-1}, ${}^{A2}V_{max}/{}^{A2}K_m$ = 0.170 l min^{-1} mg^{-1}, as well.

(*R*)- and (*S*)-PMPA, PMEDAP, (*R*)-PMPDAP and (*S*)-PMPDAP were studied as potential substrates for AK2 isoenzymes (proteins A_1 and A_2). The interaction of the above mentioned nucleoside phosphonates with both proteins A_1 and A_2 is nearly the same. For this reason only kinetic data concerning protein A_1 are presented. The time course of phosphorylation of selected compounds was compared with that of the natural substrate AMP and other acyclic nucleoside phosphonates, PMEA and (*S*)-HPMPA. The

TABLE II
Phosphate donors for purified AMP kinase[a]

Phosphate donor	Relative enzyme activity, %		
	Protein A_1	Protein A_2	Chicken muscle myokinase
ATP	100	100	100
dATP	102	101	95
GTP	17	12	12
dGTP	10	6	10
CTP	32	22	15
dCTP	14	10	28
UTP	61	66	15
dTTP	9	6	7

[a] NTP as P_i donor, AMP as P_i acceptor. The reaction mixture contained A_1 (0.318 mU) or A_2 (0.252 mU) or chicken muscle myokinase (0.02 mU), 3 mM NTP and incubation was carried out at 37 °C for 5 min.

phosphorylation pattern shown in Fig. 4a demonstrates that (*R*)-PMPA (curve *3*) is phosphorylated more efficiently than PMEA (curve *4*) but with lower efficiency compared to (*S*)-HPMPA (curve *2*). It should be noted that ADP formation (curve *1*) comparable with the phosphorylation of ANPs (curves *2*–*5*) was achieved at 1 000-fold lower concentration of protein A_1. 2,6-Diaminopurine derivatives PMEDAP, (*R*)-PMPDAP and (*S*)-PMPDAP are very poor substrates but their phosphorylation is demonstrable (Fig. 4b, curves *6*, *7*, *8*). The phosphorylation of PMEDAP, the best substrate of the compounds mentioned, proceeded with 1.7–2.4-fold higher efficiency in comparison with both enantiomers of PMPDAP; on the other hand, the phosphorylation is twice less efficient than that of PMEA (Table IV). It is evident that (*S*)-HPMPA is the best phosphonate substrate for AK2 isoenzyme. Kinetic data on the substrate activity (Table IV) show that the relative efficiency of the phosphorylation reaction of the individual ANPs varied from 3 to 100 due to substantially different values of V_{max}. To complete our study, we have also examined the phosphorylation of all phosphonate analogues by myokinase from chicken muscle – the representative of AK1 isoenzyme. However, in this case we did not detect any phosphorylation (data not shown).

On the contrary to guanine ANPs which exhibit both the substrate and inhibitory activity towards GMP kinase isoenzymes[26] we did not find any

TABLE III
Identification of purified AMP kinase

Enzyme	Recovery of enzyme activity, %			
	50 μM DNTB	30 nM Ap_5A	1 mM NEM	1 mM NEM[a] and 30 nM Ap_5A
Protein A_1	80	99	86	83
Protein A_2	88	94	87	81
Chicken muscle myokinase	7	58	1	–[b]

[a] The enzyme was pretreated with NEM at 25 °C for 1 h before the reaction assay started. [b] Not determined.

TABLE IV
Substrate activity of 9-[2-(phosphonomethoxy)alkyl]purines towards mitochondrial AMP kinase[a]

Substrate	K_m mmol l^{-1}	V_{max} nmol min^{-1} mg^{-1}	10^6 V_{max}/K_m l min^{-1} mg^{-1}	Relative efficiency of reaction[b], %
(*S*)-HPMPA	1.41	38.10	27	100
(*R*)-PMPA	1.05	8.80	8.4	31.11
(*R*)-PMPDAP	0.80	0.72	0.9	3.33
(*S*)-PMPDAP	0.50	0.69	1.4	5.18
PMEA	0.97	4.03	4.2	15.56
PMEDAP	0.67	1.55	2.3	8.52

[a] Protein A_1. [b] 100 $^{analogue}V_{max}/^{analogue}K_m/^{(S)\text{-HPMPA}}V_{max}/^{(S)\text{-HPMPA}}K_m$.

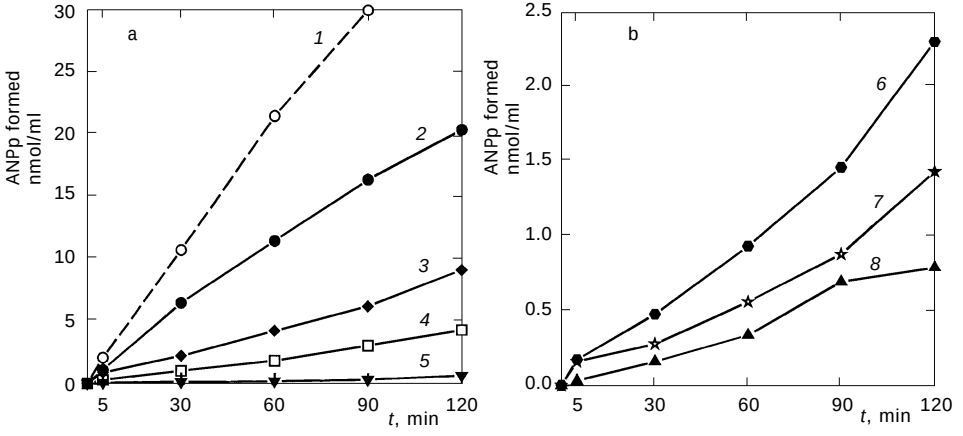


FIG. 4
Time course of phosphorylation of (phosphonomethoxyalkyl)purines catalyzed by AMP kinase (protein A_1). Reaction mixture contained 40 mM HEPES (Na^+), pH 8.0, 1 mM $MgCl_2$, 0.2 mM ATP, 2 mM 2-sulfanylethan-1-ol, 5 mM creatine phosphate, creatine kinase (50 μg ml^{-1}), 0.05 mM [U-^{14}C]-AMP and 7.7 μU of A_1 (dotted line) or the corresponding 0.05 mM [^{3}H]-(phosphonomethoxyalkyl)purine and 9.24 mU of A_1. The reactions were started by A_1 addition. Products formed a: *1* ADP, *2* (*S*)-HPMPAp, *3* (*R*)-PMPAp, *4* PMEAp, *5* (*S*)-PMPAp; b: *6* PMEDAPp, *7* (*S*)-PMPDAPp, *8* (*R*)-PMPDAPp

inhibitory effects of adenine and 2,6-diaminopurine ANPs on A_1 and A_2 proteins, as well.

DISCUSSION

Some time ago, Merta *et al.*[25] published the isolation of AMP (dAMP) kinase from leukemic L1210 cells as a dimeric protein (MW 68 kDa) which phosphorylated PMEA and (*S*)-HPMPA to their diphosphates; however, they were unable to separate AMP and NDP kinase activities. The purified protein exhibited two polypeptide bands on sodium dodecylsulfate polyacrylamide gel (MW 40 and 28 kDa, respectively). This result is not compatible with the published data concerning monomeric structure of highly purified AMP kinases from different sources[45]. The authors did not determine the type of AMP kinase isoenzyme responsible for the phosphorylation mentioned above.

The isolation procedure described in this paper enabled us to obtain AMP kinase from L1210 cells free of the NDP kinase activity and exhibiting a single polypeptide band on silver-stained sodium dodecylsulfate polyacrylamide gel (Fig. 2). The occurrence of oligomers visible on gradient gel under nondenaturing conditions (Fig. 3) is most probably due to the presence of 2-sulfanylethan-1-ol[46]. After chromatofocusing, the highly purified proteins, denoted A_1 and A_2, exhibit the same behaviour as to their kinetic constants (see Results), donor requirements (Table II), and inhibition with DTNB, NEM and Ap_5A (Table III). It is well known that AK1 is an SH enzyme, which is inhibited by NEM, while AK2 is not[42]. Therefore, the AK2 activity is defined as the activity remaining after the NEM treatment. From Table III it is evident that purified proteins A_1 and A_2 are of AK2 type. This conclusion was confirmed by the experiments with DTNB and Ap_5A. Under the experimental conditions used, our purified AMP kinase activity was not susceptible to Ap_5A, a typical competitive inhibitor of muscle adenylate kinase[39]. We suggest that the proteins A_1 and A_2 are two forms of the AK2 isoenzyme from L1210 cells. Similarly two forms of native rabbit muscle adenylate kinase (denoted N_1 and N_2) were found[47,48].

Our data present a complex phosphorylation study of (phosphonomethoxy)alkyl derivatives of adenine, *i.e.* of both enantiomers of PMPA, (*S*)-HPMPA and PMEA, as well as of 2,6-diaminopurine derivatives PMEDAP, (*S*)- and (*R*)-PMPDAP by mitochondrial AK2 isoenzyme from L1210 cells. In accordance with literature data[25,31], (*S*)-HPMPA is the best substrate (Fig. 4a, curve *2*) and exhibits higher efficiency than (*R*)-PMPA and PMEA (threefold and seven-fold, respectively). A substantially different phosphorylation

pattern of both the PMPA enantiomers by AK2 kinase isoenzyme from L1210 cells (Fig. 4a, curves *3* and *5*) indicates a lower affinity of (*S*)-PMPA for the AMP-binding site of this isoenzyme[49]. This enantiomeric preference contrasts with GMP kinase isoenzymes from L1210 cells; of guanine (phosphonomethoxy)alkyl derivatives, (*R*)-PMPG, the guanine counterpart of (*R*)-PMPA, was the best substrate[26]. This difference may be explained by distinct properties of the acceptor binding site of these enzymes[49,50].

With AK2 isoenzyme from L1210 cells, we have detected low but demonstrable phosphorylation of both the PMPDAP enantiomers, particularly of PMEDAP (Fig. 4b, curve *6*). This again probably reflects different affinity of these two groups of analogues to the binding site. It appears that the presence of the amino group in position 2 of the purine ring affects the ability of these compounds to interact with the active site and to act as substrates for AK2 isoenzyme.

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