ISOENZYMES OF GMP KINASE FROM L1210 CELLS: ISOLATION AND CHARACTERIZATION

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Guanylate kinase from a mouse leukemic L1210 cells has been purified nearly to homogeneity for the first time. It consists of five isoenzymes with p*I* 5.95, 5.50, 5.08, 4.83 and 4.51, respectively. The native enzyme is a monomer with a relative molecular mass 25 000. The kinetic constants K_m, V_{max} and k_{cat}/K_m of isoenzymes were estimated. The purified GMP kinase is absolutely specific to GMP and/or dGMP as phosphate acceptor but has a broad specificity to nucleoside 5′-triphosphates as phosphate donors.

Key words: GMP kinase; Isoenzymes; Nucleotide kinase; Phosphorylation; Nucleotides; L1210 cells; Enzymes, isolation; Phosphates.

The properties of enzymes which phosphorylate guanine nucleotides are of interest for several reasons: GTP is a substrate for RNA polymerase and is required for initiation of translation; GDP is a substrate for pyruvate kinase and ribonucleotide reductase; dGTP is a substrate for DNA polymerases; the phosphorylation of guanine nucleotide analogs is crucial for their biological activity.

Nucleoside monophosphokinases (NMP kinases) catalyze the reversible transfer of the terminal phosphoryl group from a nucleoside triphosphate (in most cases ATP) to nucleoside monophosphates. In mammalian tissues, at least four distinct NMP kinases have been identified so far[1]: adenylate kinase (AMP kinase, AK) (ref.[2]), guanylate kinase (GMP kinase, GK) (ref.[3]) and pyrimidine nucleoside monophoshokinases (dTMP kinase, CMP-UMP kinase) (ref.[4]).

While the extensive study of adenylate kinases from various sources[2] performed over many years resulted in a detailed picture of the tertiary structures and the mechanism of this enzyme family, much less is known about guanylate kinases (ATP: GMP phosphotransferases, EC 2.7.4.8) in spite of their function in the biosynthesis of GTP and dGTP. Guanylate kinase is

also believed to play an essential role in the so-called cGMP cycle[5,6] regulating the supply of guanine nucleotides to components of signal transduction pathways, possibly $p21^{ras}$ and other G-proteins in the cell cycle control mechanism[7,8].

Guanylate kinase activity, catalyzing the reversible reaction

$$Mg^{2+}\cdot ATP + (d)GMP = Mg^{2+}\cdot ADP + (d)GDP$$

was first described by Klenow and Lichtler[9]. Eukaryotic guanylate kinases are small monomeric proteins ranging in size of relative molecular mass from 18 500 to 24 000 (refs[5,10–14]). Unlike the eukaryotic enzymes, the *Escherichia coli* GMP kinase is multimeric, and ionic conditions dictate its protomeric state; under low ionic conditions, it appears to be a tetramer, while under high ionic conditions it is a dimer[15,16].

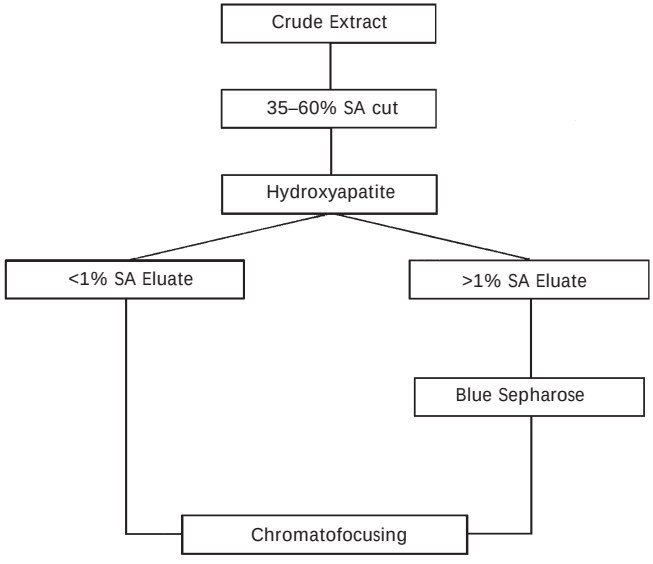
The cancerostatic[17] and antiviral effects of some purine analogs[18] led to partial or complete purification and characterization of GMP kinase from different sources. In an electrophoretically pure form it has been prepared from yeast[11,12], *E. coli* (refs[15,19]), bovine retinas and rod outer segments[5], human erythrocytes[20–23], porcine brain[24] and artichoke[25]. Guanylate kinases from rat liver[13,20], calf thymus[26] and Sarcoma 180 ascites cells[10] have been isolated in undefined purity. Agarose gel electrophoresis and isoelectric focusing of the erythrocytic GMP kinase revealed the presence of four isoenzymes with p*I* values of 4.9, 5.1, 5.4 and 5.8 (refs[20,23]), whereas only one enzyme activity was found in porcine brain GMP kinase (p*I* 5.6) (ref.[24]). Cloning and expression of the genes for GMP kinase as well as its amino acid sequence for *E. coli* (ref.[16]), yeast[27–29], porcine brain[24], bovine retinas[6] and human genome[30] were also described.

The yeast GMP kinase (GKy) is the best characterized GMP kinase both at the genetic and biochemical level[2,16,28,29]. GKy has also been crystallized as a complex with GMP, and its three-dimensional structure has been determined in 20 nm resolution[14,31,32]. The crystal structure confirms that it is a monomer with an ATP-binding site similar to that of adenylate kinase and other ATP- and GTP-binding proteins. Although its size, function, and ATP-binding site are similar to adenylate kinase, the binding of GMP by guanylate kinase is much different from the binding of AMP by adenylate kinase[14,31]. Among the adenylate kinases, the small variant of pig AK1 seems to resemble guanylate kinase most closely.

In the present study we describe the isolation of highly purified guanylate kinase from mouse leukemic L1210 cells. Physical, chemical and catalytic properties are discussed in comparison with the data for the corresponding enzymes isolated from other sources.

RESULTS AND DISCUSSION

Guanylate kinase catalyzing the phosphorylation of GMP to GDP has been purified from leukemic L1210 cells using a four-step purification procedure (Scheme 1, Table I). Due to the presence of NDP kinase in the crude cell extract, we have found after ammonium sulfate (SA) cut (35–60%) the phosphorylation of GMP to GTP in the presence of ATP. Subsequent chromatography on the hydroxyapatite column (Fig. 1) revealed only partial separation of GMP and NDP kinase activities. Stepwise elution of the GMP kinase activity with an increasing concentration of ammonium sulfate gives better results. The active fractions with the concentration of ammonium sulfate lower than 1% were nearly free of NDP kinase activity. Due to the low content of proteins, these active fractions had to be immediately concentrated using Centriprep 10, frozen in liquid N_2, stored at –78 °C and later directly used for chromatofocusing. The other active fractions from hydroxyapatite chromatography (with the concentration of ammonium sulfate higher than 1%) contained GMP kinase and NDP kinase as well.



SCHEME 1

TABLE I
Purification of L1210 GMP kinase isoenzymes[a]

Purification step	Total protein mg	Enzyme activity mU	Specific activity mU mg^{-1}	Fold purification
Crude extract	387.2	1 249.0	3.2	1
35–60%SA	149.9	1 153.0	7.7	2.4
Hydroxyapatite	56.3	921.8	16.4	5.1
Blue Sepharose	7.2	502.4	69.8	21.8
Isozyme 5.95[b]	0.131	78.0	595.4	186.1
Isozyme 5.50[b]	0.168	242.0	1 440.5	450.1
Isozyme 5.08[b]	0.128	59.0	460.9	144
Isozyme 4.83[b]	0.048	5.2	108.3	33.8
Isozyme 4.51[b]	0.042	5.2	123.8	38.7

[a] Estimated from one purification run (7 g of frozen cells). [b] Products of the chromatofocusing.

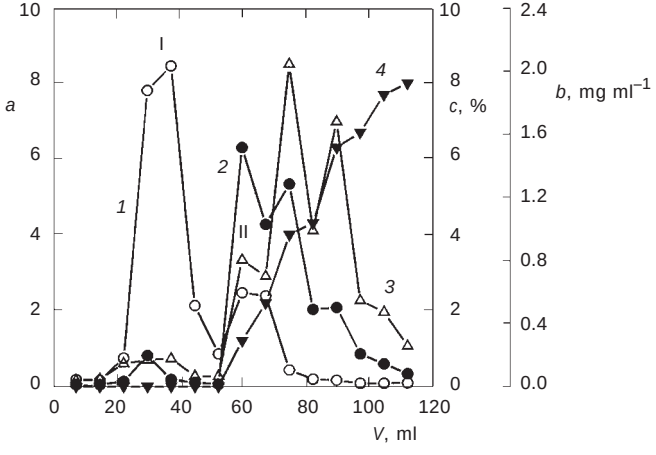


FIG. 1
Elution profile of GMP kinase on hydroxyapatite column. *1* Enzyme activity of GMP kinase, *2* enzyme activity of NDP kinase, *3* protein concentration, *4* concentration gradient of ammonium sulfate. GMP kinase and NDP kinase activities *a* are expressed as nmol min^{-1} ml^{-1} of GDP and/or GTP formed, peak II was pooled for the affinity purification step; *b* concentration of protein; *c* concentration of ammonium sulfate

To separate these enzyme activities, we have attempted to apply the chromatography on DEAE-cellulose, phosphocellulose, phenyl-Sepharose, Sephacryl 300 and Blue Sepharose. Only the Blue Sepharose affinity column was shown to be efficient for separation of GMP and NDP kinase activities (Fig. 2). Concentrated fractions from several Blue Sepharose columns containing enzyme activity were used for chromatofocusing. We have found that GMP kinase of leukemia L1210 cells consists of five isoenzymes (Fig. 3, Table I) with values of p*I* 5.95, 5.50, 5.08, 4.83 and 4.51. This procedure allowed us to purify GMP kinase near to homogeneity. Specific activity of the purified isoenzymes in terms of the GMP phosphorylation substantially increased in comparison with the crude cell extract. The highest specific activity was found for isozyme 5.5 (450-fold purified, Table I), while both isozymes 4.83 and 4.51 which make minor part of GMP kinase preparation, showed only very low specific activity. Moreover, the latter isoenzyme was losing its activity even in the presence of BSA (bovine serum albumin) at –78 °C (the remaining four isoenzymes are stable for more than one year). For this reason, we have not performed kinetic experiments with the isozyme 4.51.

The purified GMP kinase from L1210 cells was free of the NDP kinase activity and exhibited a single polypeptide band on silver-stained sodium

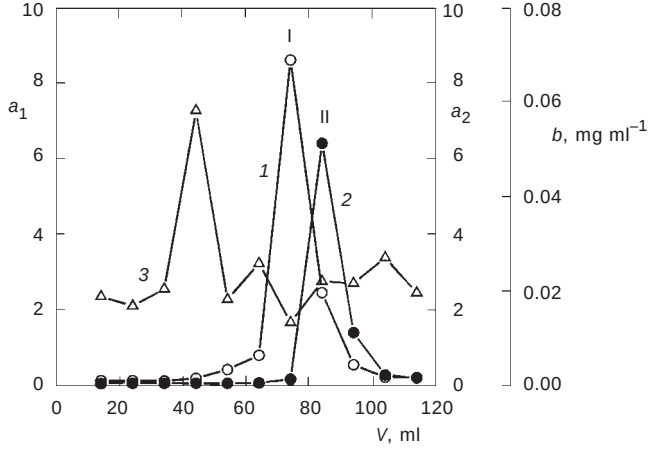


FIG. 2

Affinity purification of GMP kinase on Blue Sepharose column. *1* Enzyme activity of GMP kinase, *2* enzyme activity of NDP kinase, *3* protein concentration. GMP kinase activity a_1 (nmol min^{-1} ml^{-1} of GDP formed) and NDP kinase activity a_2 (nmol min^{-1} ml^{-1} of GTP formed), peak I was pooled for the chromatofocusing purification step; *b* concentration of protein

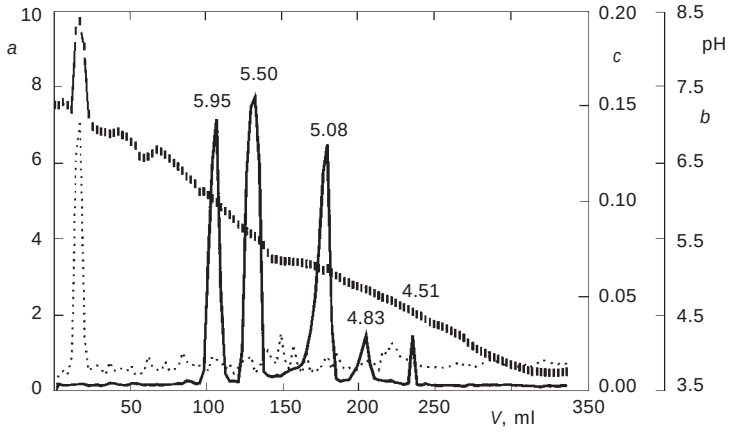


FIG. 3

Chromatofocusing of prepurified GMP kinase. pH gradient (*b*) is tickmarked, enzyme activity *a* expressed as GDP formed (nmol min^{-1} ml^{-1}; solid line), *c* concentration of proteins (mg ml^{-1}; dotted line)

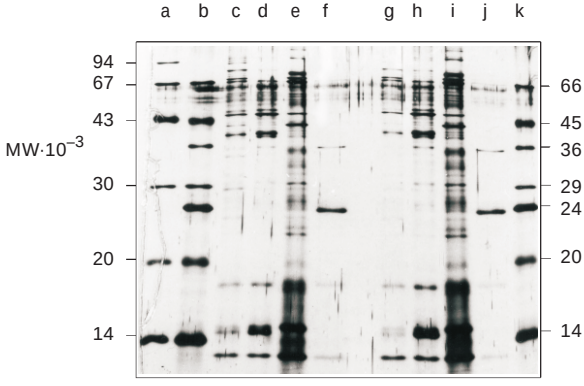


FIG. 4

Purification of GMP kinase – SDS–PAGE pattern. Lanes (from left to right): LMW calibration kits a (phosphorylase b 94 000, BSA 67 000, ovalbumin 43 000, carbonic anhydrase 30 000, soybean trypsin inhibitor 20 000, α-lactalbumin 14 000); b, k (albumin 66 000, ovalbumin 45 000, glyceraldehyde-3-phosphate dehydrogenase 36 000, carbonic anhydrase 29 000, trypsinogen 24 000, soybean trypsin inhibitor 20 100, α-lactalbumin 14 200); c, g 35–60% ammonium sulfate cut; d, h peak II from hydroxyapatite; e, i peak I from Blue Sepharose column; f, j isozyme 5.95 of GMP kinase

dodecyl sulfate polyacrylamide gel (Fig. 4) with the relative molecular mass 25 000. This is similar to that of GMP kinase from yeast[11], porcine brain[24], retinas and rod outer segments[5]. After gradient polyacrylamide gel electrophoresis under nondenaturing conditions (in the presence of dithiothreitol (DTT)), the GMP kinase phosphorylating activity was found to migrate as oligomers (Fig. 5). Most probably due to the presence of DTT (ref.[33]), the monomer was detected as minor component only.

We have found that all isoenzymes of GMP kinase from leukemic L1210 cells are absolutely specific for guanine nucleotides (GMP and dGMP) as phosphate acceptor (data not shown). In contrast, the binding site for the phosphate-donating nucleoside 5′-triphosphate exhibits much less specificity (Table II). While the purine nucleotide GTP could replace ATP with less than 18% efficiency, pyrimidine nucleotides were more efficient donors. For example, in the presence of CTP, the isozyme 5.5 catalyzes the phosphorylation of GMP with 59% efficiency of ATP. Similar results were found for adenylate kinase from the thermoacidophile *Sulfolobus acidocaldarius* (ref.[34]). In general, the donor efficiency of nucleotide 5′-triphosphates decreases in the order ATP(dATP) > CTP(dCTP) > UTP > GTP(dGTP) = dTTP.

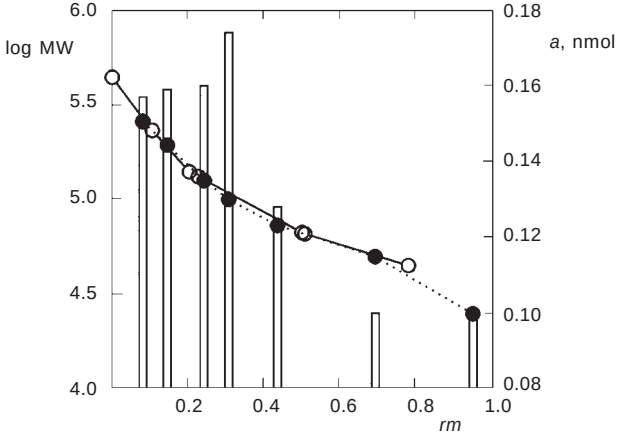


FIG. 5

Gradient PAGE pattern of GMP kinase isozyme 5.50. Relative mobility, *rm*, molecular weight standard proteins (○) (from left to right: ferritin, catalase, lactate dehydrogenase, albumin dimer, albumin 67 000, albumin 66 000, ovalbumin), position of GMP kinase isoenzyme 5.50 protomer and multimers (●), respectively (from right to left: monomer, dimer, trimer, tetramer, pentamer, octamer, dodekamer). GMP kinase activity, *a*, expressed in nmol of GDP formed in the reaction mixture (150 min, 30 °C)

Kinetic constants K_m were nearly identical with the four isoenzymes (Table III); similar observation was made with isoenzymes from human erythrocytes[23]. The values are comparable with K_m values estimated for yeast[12], retinas[5] and rat liver[20]. On the other hand, the V_{max} values of isoenzymes tested, differed within two orders of magnitude (for isozyme 5.5 and 4.83) (Table III). The ratio k_{cat}/K_m, which reflects the catalytical efficiency of the enzyme is

TABLE II
Substrate specifity of L1210 GMP kinase isoenzymes[a]

NTP as P_i donor	Relative enzyme activity, %			
	isozyme 5.95[b]	isozyme 5.50[b]	isozyme 5.08[b]	isozyme 4.83[b]
ATP	100	100	100	100
dATP	102	102	118	108
GTP	7	9	11	18
dGTP	10	12	12	22
CTP	43	59	32	32
dCTP	42	52	29	31
UTP	21	22	21	27
dTTP	7	9	10	19

[a] GMP as P_i acceptor. The reaction mixture (20 μl) contained: 0.046 mU of 5.95 isozyme, 0.108 mU 5.50 isozyme, 0.035 mU 5.08 isozyme and/or 0.009 mU 4.83 isozyme. The reaction mixture contained 4 mM NTPs and incubation was carried out for 30 min at 30 °C.

TABLE III
Kinetic properties of L1210 GMP kinase isoenzymes[a]

GMP kinase isoenzyme	K_m	V_{max}	k_{cat}/K_m
	μmol l^{-1}	μmol min^{-1} mg^{-1}	s^{-1} mol^{-1} l
Isozyme 5.95	30.9	0.732	77.6
Isozyme 5.50	30.2	2.481	305.7
Isozyme 5.08	27.5	0.729	95.7
Isozyme 4.83	42.7	0.094	6.8

[a] See Experimental for details.

practically equal for isozymes 5.95 and 5.08, higher for isozyme 5.5 and lower for 4.83. This relation probably reflects a different function of these GMP kinase isoenzymes *in vivo*.

EXPERIMENTAL

Materials

[U-^{14}C]-GMP (16.5 GBq mmol^{-1}) was synthesized in the Institute for Research, Production and Uses of Radioisotopes in Prague (Czech Republic). Proteinase inhibitors (pepstatin, leupeptin, aprotinin, bestatin), PMSF (4-methylbenzenesulfonyl fluoride), DTT and BSA were products of Sigma. Polybuffer exchanger PBE 94, polybuffers PB 74 and PB 96 for chromatofocusing, molecular weight calibration kits (HMW and LMW), Blue Sepharose and columns PD-10 were purchased from Pharmacia P-L Biochemicals (Sweden). Polygram® Cel 300 PEI/UV$_{254}$ for TLC chromatography was from Macherey–Nagel (Germany). Hydroxyapatite was prepared according to Schomburg and Grosse[35]. 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-sulfanylethanol and stored at –78 °C.

Concentration of the Enzyme Fractions

The enzyme fractions were concentrated using Centriprep-10 (2 000 g, 90 min) and Centricon-10 (4 000 g, 60 min).

Enzyme Assay

Guanylate kinase activity was measured by radiochemical assay. The standard reaction mixture (20 µl) contained 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 4 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM [U-^{14}C]-GMP (0.99 GBq mmol^{-1}) and 10 µl of tested fractions. The reactions were carried out at 30 °C for 10 min and stopped by spotting 5 µl onto a polyethyleneimine-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 system 0.8 M CH_3COOH and 0.8 M LiCl. 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 the phosphorylation of 1 µmol of GMP per minute under the conditions of the standard assay. Specific activity of an enzyme is expressed in units per milligram of protein.

Kinetic Experiments

Kinetic constants (K_m and V_{max}) were determined from the Lineweaver–Burk plot using varying concentration of GMP (15 concentrations, 2.5–200 µM) at fixed concentration of ATP

(1 mM saturating concentration) and an appropriate aliquot of GMP kinase isoenzyme (isozyme 5.95, 9.3 μU; isozyme 5.50, 10.8 μU; isozyme 5.08, 8.4 μU; isozyme 4.83, 18.6 μU). Data were evaluated by the nonlinear regression method based on results from four independent experiments. Reactions were carried out at 30 °C for 10 min. The kinetic constant k_{cat} was calculated from the equation $k_{cat} = V_{max}/E_0$, where E_0 is the final enzyme concentration (30 °C, time interval 1–5 min, at various amounts of individual isoenzyme (0.01–0.02 mU), concentration of GMP was ≈5 K_m) (refs[36–38]).

Denaturing Gel Electrophoresis of Proteins

SDS–PAGE system according to Laemmli[39] (15% polyacrylamide) was used for the analysis of the purified GMP kinase isoenzymes. Samples were heated at 95 °C for 10 min (in some cases after concentration by evaporation in vacuum) in 30 mM Tris-HCl, pH 6.8, 5% SDS, 5% glycerol (v/v) and 5% 2-sulfanylethanol. LMW calibration kit was used as a protein standard. The gels were stained with silver[40].

Native Gel Electrophoresis of Proteins

Native gradient slab gels (4–30%) were run to equilibration (2 500 V h) on a 2050 MIDGET-Electrophoresis Unit (LKB); the gels had been preelectrophoresed for 30 min in TBE buffer, pH 8.3, containing 4 mM DTT. LMW and HMW calibration kits were used as protein standards. One part of the gel was stained with silver[40] and the other was cut into 2-mm slices. GMP phosphorylating activity was determined after extraction of each slice with an assay mixture which contained (150 μl): 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 100 μM ATP, 200 μM $MgCl_2$, 2 mM DTT, 30 μM [U-^{14}C]-GMP (3.3 GBq mmol^{-1}) and BSA (0.4 mg ml^{-1}).

Protein Assay

Protein concentration was determined by the method of Bradford[41] with bovine serum albumin as standard.

Buffers

Buffer A: 100 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, bestatin (40 μg ml^{-1}), leupeptin (2 μg ml^{-1}), pepstatin A (4 μg ml^{-1}), aprotinin (2 μg ml^{-1}).
Buffer B: 5 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM PMSF.
Buffer C: 2 mM HEPES, pH 7.0, 1 mM DTT.
Buffer D: 10 mM HEPES, pH 7.0, 50 mM KCl, 1mM $MgCl_2$, 1 mM DTT.
Buffer E: 25 mM Bis-Tris-HCl, pH 7.4.
Buffer F: Polybuffer 74-HCl, pH 4.0.
Buffer TBE: 90 mM Tris-boric acid, pH 8.3, 2 mM EDTA.

Purification Procedure

All procedures were performed at 4 °C, GMP kinase was purified from the crude cell extract by chromatography on hydroxyapatite and Blue Sepharose columns and finally by chromatofocusing.

Crude cell extract. The frozen L1210 cells (27 g) were thawed and suspended in 108 ml of buffer A and GMP kinase activity was extracted from the cells by freeze-thawing (three

times) followed by homogenization in Dounce tissue grinder (Wheaton, 15 strokes of pestle B). The final suspension was centrifuged for 30 min at 20 000 g and the supernatant was used for next purification steps.

Ammonium sulfate (35–60%) cut. The clear supernatant was stepwise precipitated with solid $(NH_4)_2SO_4$ (SA). The precipitate formed at 35–60% saturation was left to stand overnight at 4 °C and stored at –20 °C (fraction I).

Chromatography on hydroxyapatite. Aliquot of fraction I was dissolved in 5 ml of buffer B, centrifuged for 30 min at 20 000 g and the clear supernatant was desalted on PD-10 columns pretreated with buffer B. Final volume 7 ml (64 mg of protein) was applied onto a hydroxyapatite column (1.5 × 8.3 cm), equilibrated in buffer B. After washing the column with two column volumes of an equilibration buffer (0.2 ml min^{-1}, fraction volume 7.5 ml), the GMP phosphorylating activity was eluted stepwise with one column volume each of buffer B containing 2, 4, 6, 8 and 10% ammonium sulfate (Fig. 1). Active fractions with the concentration of ammonium sulfate lower than 1% (peak I, 15 ml) were immediately concentrated using Centriprep 10 to the final volume *ca* 1 ml and stored at –78 °C. The other fractions (peak II, eluted with the concentration of ammonium sulfate 1–7%) were precipitated with solid ammonium sulfate to 70% saturation and stored at –20 °C (fraction II).

Chromatography on Blue Sepharose. Aliquot of fraction II was dissolved in 5 ml of buffer C and centrifuged for 20 min at 20 000 g. The clear supernatant was desalted on PD-columns and the eluate (final volume 7 ml, 33 mg protein) was applied onto a Blue Sepharose column (1.4 × 6.5 cm) equilibrated with buffer C (flow rate 0.25 ml min^{-1}); 5-ml fractions were collected and tested for guanylate kinase activity. After washing the column with two column volumes of buffer D, the enzyme activity was eluted with 1 mM ATP in the same buffer (three column volumes) (Fig. 2). The active fractions were pooled (peak I), concentrated using Centriprep 10 and stored at –78 °C (fraction III).

Chromatofocusing. Chromatofocusing was performed on a polybuffer exchanger PBE 94 column (0.8 × 40 cm) equilibrated with buffer E. After elution of column with 6 ml of buffer F, fraction III (6 ml, 5–15 mg of protein) was applied onto the column and the pH gradient in the column was created by elution with the same buffer F (0.2 ml min^{-1}, 3 ml fractions; Fig. 3). The active fractions were pooled and concentrated (using either Centriprep 10 or Centricon 10, respectively) or stabilized by BSA (final concentration, 0.4 mg ml^{-1}). Aliquots of fractions were immediately frozen in liquid N_2 and stored at –78 °C.

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