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Adenosine Triphosphate–Guanosine 5′-Phosphate Phosphotransferase

IV. Isozymes in Human Erythrocytes and Sarcoma 180 Ascites Cells

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SUMMARY

Four isozymes of guanylate kinase (ATP:GMP phosphotransferase, EC 2.7.4.8) with isoelectric points (pI) of 4.9, 5.1, 5.4, and 5.8 in human erythrocytes and two isozymes with isoelectric points of 5.2 and 5.5 in Sarcoma 180 ascites cells have been identified and separated by two electrophoretic techniques. The molecular weights of the isozymes range from 18,500 to 24,000. GMP, dGMP, and 8-azaguanosine 5′-phosphate serve as substrates for each of the guanylate kinase isozymes. Three of the erythrocytic guanylate kinase isozymes (pI 4.9, 5.1, and 5.4) react with IMP with a velocity of about 0.4%, while with the fourth isozyme (pI 5.8) the velocity is about 1% of that seen with GMP. The analogue nucleotide, 6-thioguanosine 5′-phosphate, behaves as a potent competitive inhibitor of all six guanylate kinase isozymes. The inhibition constant (K_i) values range from 7.5×10^{-5} to 2.1×10^{-4} M.

INTRODUCTION

In earlier studies reported from this laboratory (1–3), ATP-GMP phosphotransferase (guanylate kinase) (EC 2.7.4.8) was identified in a number of tissues, including hog brain, Sarcoma 180 cells, and human erythrocytes. It was postulated that this enzyme plays a significant role in cancer chemotherapy because it appears to be the primary and perhaps the only mechanism in the cells for converting GMP and dGMP to the diphosphate nucleotide level and, in addition, the enzyme is strongly inhibited by the analogue nucleotide, 6-thioguanosine 5′-phosphate. When the anticancer and immunosuppressive analogue, 6-thioguanine, was incubated with isolated tumor cells, the analogue nucleotide, 6-thioGMP,[1] accumulated to levels in the range of 0.1 mM, a concentration much greater than the steady-state concentration of GMP in these cells (4–6). Since little or no 6-thioguanosine di- or triphosphate was detected, a metabolite block at the level of guanylate kinase appears to occur in these cells as predicted from observations on the isolated enzyme (2, 7).

When radioactive 6-thioguanine was administered to various tumor systems, small amounts of 6-thioguanine were incorporated into the nucleic acids (8–11), suggesting the existence of isozymes of guanylate kinase that can react efficiently with 6-thioGMP as a substrate (7). Also, the possibility exists that certain cells resistant to the action of 6-thioguanine possess a guanylate kinase isozyme that is not inhibited by 6-thioGMP.

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[1] The abbreviations used are: 6-thioGMP, 6-thioguanosine 5′-phosphate; 8-azaGMP, 8-azaguanosine 5′-phosphate.

For these reasons, a search was undertaken for the existence of isozymes of guanylate kinase. The present report demonstrates the occurrence of four isozymes of guanylate kinase in human erythrocytes and two isozymes in Sarcoma 180 ascites cells. All six isozymes are inhibited to a similar degree by 6-thioGMP. A preliminary report of this work has been presented (12).

MATERIALS AND METHODS

The various nucleotides, NADP, NADH, phosphoenolpyruvate, and 5-phospho-α-D-ribosyl 1-pyrophosphate were purchased from P-L Biochemicals. 8-azaGMP was generously supplied by the Cancer Chemotherapy Service Center. The concentration of 8-azaGMP was determined by its molar absorbance at pH 7.4 ($\epsilon = 10.5 \times 10^3$ at 257 nm) (13, 14). 6-Thioguanine was obtained from Schwarz BioResearch. Suspensions of adenylate kinase (grade III), pyruvate kinase (type II), lactate dehydrogenase (type II) (all from rabbit muscle), hexokinase (type c-300), glucose 6-phosphate dehydrogenase (type VII) (from yeast), phenazine methosulfate, and agarose were purchased from Sigma Chemical Company. Peroxidase from horseradish, DEAE-cellulose (Cellex-D), and dithiothreitol were purchased from Calbiochem. The dye 3-(4,5-dimethylthiazolyl - 2) - 2,5-diphenyltetrazolium bromide was obtained from Nutritional Biochemicals Corporation. Cytochrome *c* and bovine plasma albumin were products of Boehringer/Mannheim and Armour Pharmaceutical Company respectively. Bio-Gel (100–200 mesh) was a product of Bio-Rad Laboratories. All other chemicals used were of the highest purity. Outdated human blood was obtained from the Miriam Hospital, Providence, R. I., and from the Red Cross in Boston.

6-ThioGMP was prepared enzymatically according to Miech *et al.* (2), except that the ratio of 6-thioguanine to 5-phospho-α-D-ribosyl 1-pyrophosphate in the reaction mixture was 1:1. The yield of 6-thioGMP compared favorably with that reported earlier (2). Immediately after preparation and isolation from a DEAE-cellulose bicarbonate column, 6-thioGMP was divided into appropriate aliquots, lyophilized, and stored under nitrogen at −20° until required for study. Immediately prior to the enzymatic studies, a solution of 6-thioGMP was prepared and its molar absorbance was determined at pH 4.1 ($\epsilon = 24.8 \times 10^3$ at 342 nm) (15).

Female mice (CF-1 strain) were obtained from Carworth Animal Farms. The initial inoculum of 6-thioguanine-sensitive Sarcoma 180 cells was generously supplied by Dr. A. C. Sartorelli, Yale University.

Tris-Maleate Electrophoretic Solution

Agarose gel electrophoresis was performed in a buffer system containing 24.2 g of Tris, 23.2 g of maleic acid, 5.85 g of tetrasodium EDTA, and 4.1 g of $MgCl_2 \cdot 6H_2O$ dissolved in 1 liter of distilled water. The pH was adjusted to 7.2 with solid NaOH. Prior to use the solution was diluted with water to a final conductivity of about 2.9 millimhos.

Enzyme Assays

A Beckman model DUR spectrophotometer equipped with a model 209 Gilford absorbance meter, a Gilford model 2000 recorder, and thermospaces was used in the spectrophotometric enzyme assays, all of which were performed at 30°.

Guanylate kinase activity was measured by recording the decrease in absorbance at 340 nm by the coupled pyruvate kinase–lactate dehydrogenase procedure described earlier (1), except that the concentration of ATP was 4.0 mM. Adenylate kinase activity was measured similarly, except that GMP was replaced by 0.1 mM AMP.

One micromolar unit of guanylate kinase or adenylate kinase is defined as the amount of enzyme that will catalyze the phosphorylation of 1 μmole of GMP or AMP per minute under the conditions of the standard assay. The phosphorylation of 1 μmole of GMP in the presence of ATP ultimately results in the oxidation of 2 μmoles of NADH because both ADP and GDP serve as substrates for pyruvate kinase (3).

Protein concentrations were determined by the method of Warburg and Christian (16).

Peroxidase activity was measured by the

method described in the "Worthington Manual" (1968) supplied by Worthington Biochemical Corporation.

Partial Purification of ATP-GMP Phosphotransferase (Guanylate Kinase)

From human blood. About 0.4 unit of the enzyme is present per milliliter of packed erythrocytes. In order to obtain substantial quantities of guanylate kinase, the initial steps of purification were carried out at the New England Enzyme Center at Tufts University Medical School, Boston, by a method described elsewhere (3), starting with 14 liters of washed human erythrocytes. This made available about 2000 units of guanylate kinase purified about 140-fold to a specific activity of 0.14 unit/mg of protein. Approximately 1000 units were purified further to a specific activity of about 2.2 units/mg of protein by DEAE-cellulose column chromatography and 65% ammonium sulfate precipitation as described earlier (3). By passing this enzyme sample through a Bio-Gel P-100 column, the specific activity was raised to 3.3 units/mg of protein. Total yield was about 150 units. This sample was used for electrophoretic studies described below.

From Sarcoma 180 cells. Sarcoma 180 ascites cells were harvested from female mice 6 days after intraperitoneal inoculation of about 5×10^6 cells/mouse. The harvested tumor cells were washed with 0.9% NaCl solution six or seven times to remove any trace of blood. The packed cells were suspended in 3 volumes of Tris-acetate buffer, 0.1 M (pH 7.5). Fifty-milliliter aliquots were sonicated for 2.5 min at 112 W of acoustic energy in an ice bath, using a Biosonic sonicator, model II. The debris was removed by centrifugation at 12,000 $\times$ *g* for 1 hr. The supernatant fluid (sample I) was treated with 2% protamine sulfate with continuous gentle stirring to remove the nucleic acids. The final ratio of protamine sulfate to protein in the supernatant fluid was 1:15. The precipitated nucleic acids were removed by centrifugation at 10,000 $\times$ *g* for 20 min. Further steps in the purification of guanylate kinase activity were similar to those used for human erythrocytes (fractionation at 40–60% saturation with ammonium sulfate, DEAE-cellulose column chromatography, and precipitation with 65% ammonium sulfate saturation). This fraction (sample II) had a specific activity of 1.1 units/mg of protein.

A portion of sample II, after dialysis against 0.005 M phosphate buffer (pH 7.5), was treated with calcium phosphate gel (17) for 30 min at a gel to protein ratio of 10:1. After centrifugation, the guanylate kinase activity remaining in the supernatant fluid (sample III; specific activity, 1.5 unit/mg of protein) was virtually devoid of adenylate kinase activity. Samples I, II, and III of guanylate kinase were used for the electrophoretic studies described below.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed on 1% agarose plates, using an apparatus supplied by Metalloglass, Inc. Samples containing about 0.005 unit of guanylate kinase were applied, and the electrophoresis was performed at 200 V for 90 min in the Tris-maleate electrophoretic solution described above.

For identification of the bands of enzymatic activity, overlay plates were prepared as follows. Sufficient agarose to yield a final concentration of 1% was added to 0.05 M Tris-chloride buffer (pH 8.0) and dissolved by gentle boiling. This solution was allowed to cool to about 45°, mixed rapidly with the indicator enzyme reaction mixture, and immediately poured into a mold formed by two glass sheets separated by a 1/16-inch-thick plastic gasket. The components of the indicator system in the overlay plate were ADP, 1 mM; GDP, 1 mM; magnesium chloride, 100 mM; glucose, 5 mM; yeast hexokinase, 0.2 unit/ml; glucose 6-phosphate dehydrogenase NADP, 0.1 mM; phenazine methosulfate, 0.01%; and 3-(4,5-dimethylthiazolyl-2)-2,5- diphenyltetrazolium bromide, 0.1%. The final volume before preparation of the mold was 20 ml. The overlay mold was allowed to solidify in the dark at 4° for about 30 min. It was next placed carefully over the electrophoretic running plate upon the completion of electrophoresis, and the two plates were incubated in close contact at 37°.

During the reaction, components of the overlay plate diffuse into the running plate. Also, the enzymes in the running plate diffuse into the overlay plate, with the result that the guanylate kinase and adenylate kinase reactions occur in both plates. As the reactions proceed, bluish pink bands of insoluble formazan are deposited in both plates. Usually incubation for 1–2 hr at 37° gives sufficient color development. After completion of incubation, the overlay and running plates are separated and the excess dye is washed away in the dark with running water. The agarose plates may be allowed to dry on glass sheets to form permanent preparations. It should be noted that this coupled reaction system detects isozymes of both guanylate kinase and adenylate kinase. However, when GDP is omitted from the indicator system, the method becomes specific for adenylate kinase. This control was included in each experiment.

Isoelectric Focusing

Electrofocusing was carried out in an LKB electrofocusing column (110 ml) in a sucrose gradient with 1 or 2% AMPHOLINE containing a mixture of ampholytes at pH 4–6 for 50–60 hr at 500 V (18).

RESULTS

Demonstration of Guanylate Kinase Isozymes in Human Erythrocytes

As shown in Fig. 1, when aliquots of hemolysates of human erythrocytes were subjected to agarose electrophoresis at pH 7.2, three distinct bands of guanylate kinase activity were identified. The overlay method employed also detects isozymes of adenylate kinase, which may be distinguished from guanylate kinase by omitting the substrate, GDP, from the overlay. In this and other experiments, four bands of adenylate kinase activity were detected. When erythrocytic

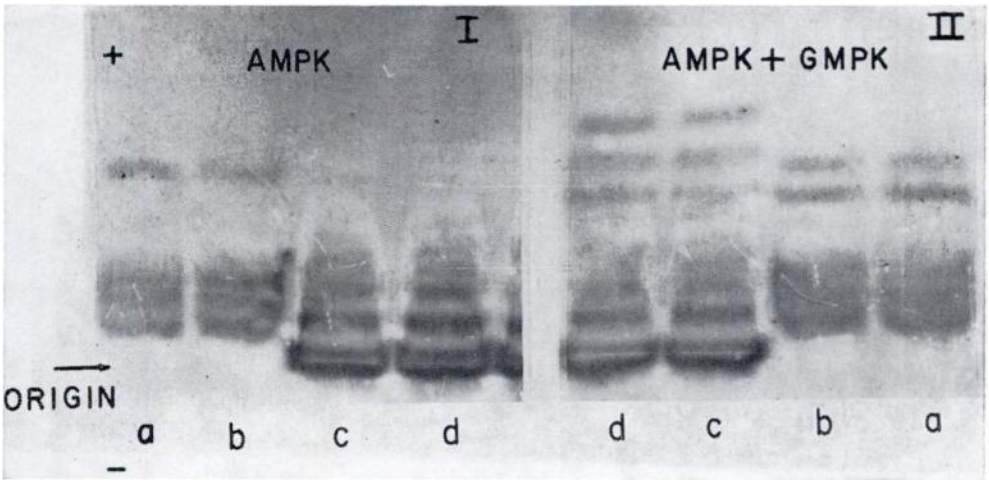

FIG. 1. *Agarose gel electrophoretic pattern of guanylate kinase (GMPK) and adenylate kinase (AMPK) in hemolysates of human erythrocytes from two individuals and from an extract of Sarcoma 180 ascites cells*

Samples (5–7 μl) from the extract of Sarcoma 180 cells (*a* and *b*) and from hemolysates of two individuals (*c* and *d*), containing about 0.005 unit of guanylate kinase activity, were applied to the agarose running plate. The electrophoresis was carried out for 90 min, at 200 V, using the Tris-maleate electrophoretic solution described under MATERIALS AND METHODS. In plate I (left) the overlay system for adenylate kinase alone was employed. In running plate II (right) an overlay method was used that detects both adenylate and guanylate kinases (see MATERIALS AND METHODS). With the hemolysates (*c* and *d*) at least four bands of adenylate kinase are observed, and three clear-cut bands of guanylate kinase (upper three bands in II*c* and *d*). With the Sarcoma 180 cells (*a* and *b*) four bands of adenylate kinase are seen (I), and one distinct band of guanylate kinase (II*a* and *b*). From differences in color intensity (not apparent in this photograph) it appeared that the upper bands in II*a* and *b* contained both adenylate and guanylate kinases.

guanylate kinase was partially purified to a specific activity of 3.3 as described under MATERIALS AND METHODS and subjected to agarose electrophoresis, the three major bands of guanylate kinase activity appeared. No bands of adenylate kinase activity were seen. As shown earlier (3), this purification procedure includes treatment of the hemolysate with calcium phosphate gel (which does not adsorb guanylate kinase) followed by elution from DEAE-cellulose (phosphate) at pH 6.8, which completely removes adenylate kinase.

In order to confirm the occurrence of guanylate kinase isozymes and to obtain sufficient amounts of each isozyme for further studies, isoelectric electric focusing procedures were employed, using Ampholine gradients from pH 4 to 6 (Fig. 2a and b). In the experiment shown in Fig. 2a, a 2% Ampholine solution was employed. After electrofocusing for 50 hr at 500 V, 1-ml fractions were collected and the enzymatic activity, pH, and absorbance at 280 nm were determined. The contents of tubes 32–70 were pooled (pH 4.7–5.9) and again subjected to isoelectric focusing after the establishment of a new sucrose gradient (Fig. 2b). The results in Fig. 2b show four distinct, completely separated peaks of guanylate kinase activity with isoelectric points (pI) of about 4.9, 5.1, 5.4, and 5.8. Aliquots from each of the four tubes of peak enzymatic activity were subjected to agarose electrophoresis followed by the overlay procedure. In each case a single, distinct band of

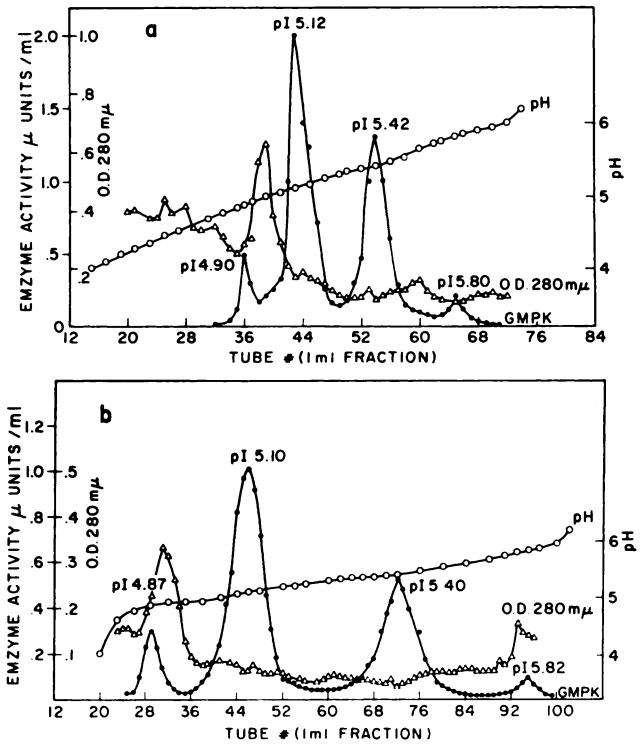

FIG. 2. *Electrofocusing profile of partially purified guanylate kinase (GMPK) from human erythrocytes*

a. About 20 units (specific activity, 3.3 units/mg of protein) of guanylate kinase were electrofocused in an electrofocusing column (110 ml) containing 2% Ampholine (pH 4–6) in a sucrose gradient. After electrofocusing for 50 hr at 500 V, 1-ml fractions were collected. Enzymatic activity, pH, and absorbance at 280 nm were determined.

b. The contents of tubes 32–70 (pH 4.7–5.9) from Fig. 2a were pooled and again subjected to electrofocusing for 60 hr at 500 V after the establishment of a new sucrose gradient. Other conditions were similar to those described for Fig. 2a.

guanylate kinase activity was observed (Fig. 3).

Guanylate Kinase Isozymes in Sarcoma 180 Ascites Cells

Aliquots of the Sarcoma 180 supernatant fluid (sample I in MATERIALS AND METHODS) were subjected to agarose electrophoresis as shown in Fig. 1. One distinct band of guanylate kinase activity was detected. A second band, probably of guanylate kinase activity, appeared to be contaminated by adenylate kinase activity. At least four bands of adenylate kinase activity were noted (Fig. 1). When a fraction of Sarcoma 180 guanylate kinase (sample III) which was free of adenylate kinase activity (described under MATERIALS AND METHODS) was subjected to agarose electrophoresis, two distinct bands of guanylate kinase activity were seen.

When the guanylate kinase in the supernatant fraction (sample I) of sonicated Sarcoma 180 cells was purified approximately 100-fold (sample II as described under MATERIALS AND METHODS) and subjected to isoelectric focusing from pH 4.0 to 6.0, the pattern shown in Fig. 4a was observed. Two peaks of guanylate kinase activity were detected, with isoelectric points of about 5.2 and 5.5. In addition, three peaks of adenylate kinase activity were observed, with isoelectric points of about 5.2, 5.6, and 5.9. The apparent superimposition of peaks of these two enzymes at about pH 5.2 suggested the possible occurrence of nonspecific mononucleotide kinase capable of reacting with either GMP or AMP. Therefore, sample II was purified further by treatment with calcium phosphate gel, as described under MATERIALS AND METHODS, yielding sample III, which was subjected to isoelectric focusing. Treatment with calcium phosphate gel removed the adenylate kinase activity completely, and now only two peaks of guanylate kinase, with isoelectric points of 5.2 and 5.5, were observed (Fig. 4b). Because of the relatively low protein concentration, it was difficult to estimate accurately the specific activities of these peaks. However, it appears that the specific activity of the pI 5.5 isozyme is at least 10 units/mg of protein, which would represent approximately a 1000-fold increase in the specific activity in comparison with the guanylate kinase activity of the sonicated cell extract. In order to confirm further the occurrence of separate activities of adenylate kinase and guanylate kinase in tubes 54–57 of Fig. 4a, the contents

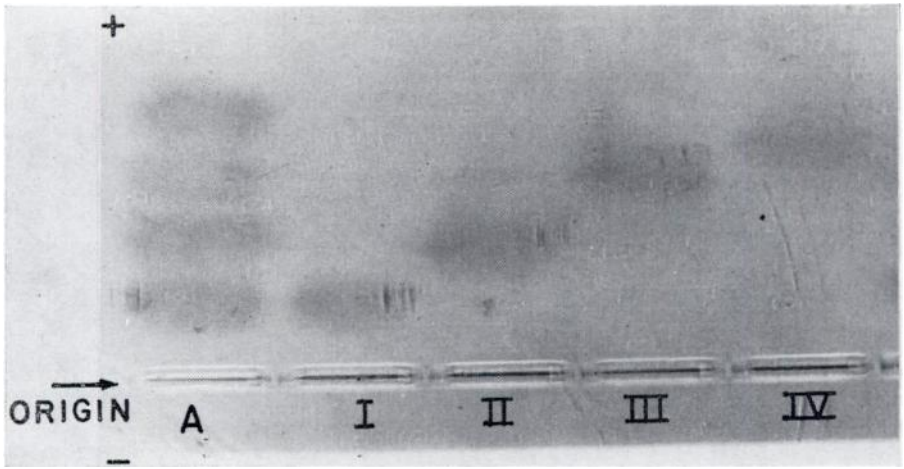

FIG. 3. *Agarose gel electrophoretogram of erythrocytic guanylate kinase isozymes separated by electrofocusing in Fig. 2b*

A sample of a mixture (*A*) of four different erythrocytic guanylate kinase isozymes, and samples I, II, III, and IV (pI 5.8, 5.4, 5.1, and 4.9, respectively) from each of the enzymatic peak tubes of Fig .2b, were subjected to electrophoresis for 80 min. Other conditions were similar to those of Fig. 1. Guanylate kinase enzymatic bands were located by the specific overlay technique as described in the text.

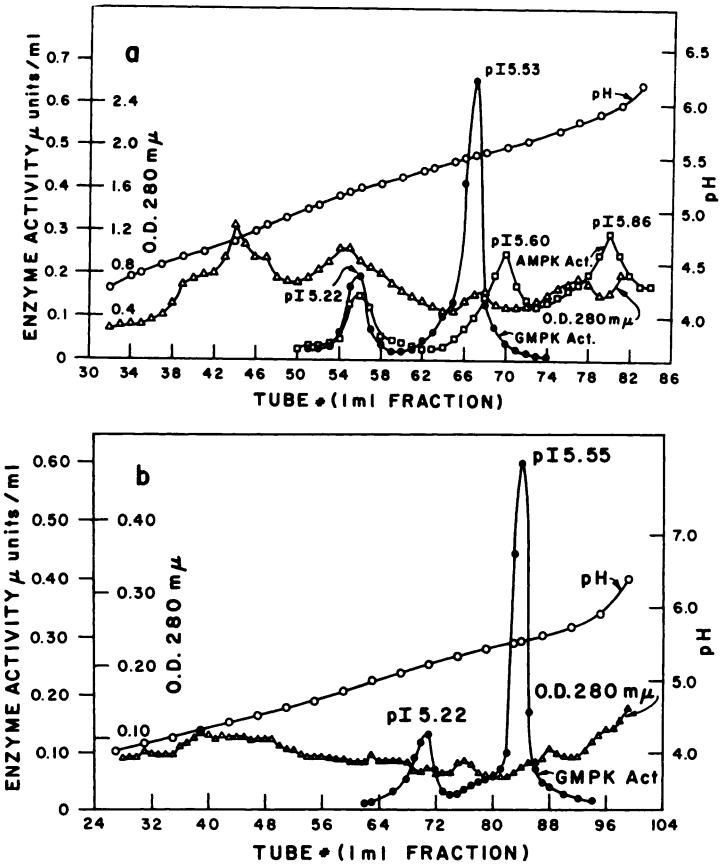

FIG. 4. *Electrofocusing profile of partially purified samples of guanylate kinase from Sarcoma 180 ascites cells*

a. About 4 units of guanylate kinase (GMPK) sample II (specific activity, 1.1 units/mg of protein) (see MATERIALS AND METHODS) containing an approximately equal amount of adenylate kinase (AMPK) activity were electrofocused in 1% Ampholine (pH 4–6) for 60 hr at 500 V. The absorbance at 280 nm, the pH, and the enzymatic activities were determined in each fraction.

b. About 3 units of guanylate kinase sample III (specific activity, 1.5 units/mg of protein) (see MATERIALS AND METHODS) were electrofocused as described for Fig. 4a.

of these tubes were pooled, concentrated, and passed through a Bio-Gel P-100 column. The activities of adenylate kinase and guanylate kinase were separated completely by this procedure. Through the use of marker proteins of known molecular weights, the adenylate kinase isozyme had a molecular weight of about 60,000, whereas that of the guanylate kinase isozyme was 19,500, which is in accordance with findings from previous studies (2).

In the experiment described below, the isozymes employed were obtained from pooled and dialyzed fractions of the two or three tubes containing the greatest enzymatic activity in the peaks of Figs. 2b and 4b.

Estimation of Molecular Weights

When an aliquot of erythrocytic guanylate kinase, purified about 3300-fold to a specific activity of 3.3 units/mg of protein, was subjected to molecular sieving in order to estimate the molecular weight by the method of Andrews (19), a broad band of guanylate kinase activity that appeared to contain two peaks was observed (Fig. 5). The guanylate kinase peak was much wider than the peaks of the marker proteins. The guanylate kinase

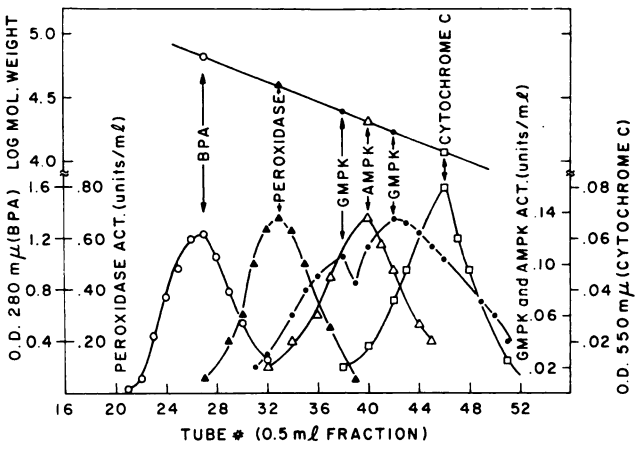

FIG. 5. *Estimation of molecular weight of erythrocytic guanylate kinase*

A mixture of the following proteins in 0.5 ml of 0.1 M Tris-acetate buffer (pH 7.5) was added to a Bio-Gel P-100 column (1.0 × 60 cm): 1 unit of guanylate kinase (GMPK) (specific activity, 3.3 units/mg of protein), 2 mg of bovine plasma albumin (BPA) (mol wt 67,000), 3 units of peroxidase (mol wt 40,000), 2 units of adenylate kinase (AMPK) (mol wt 21,000), and 2.5 mg of cytochrome *c* (mol wt 13,000). Elution was carried out with 0.1 M Tris-acetate, pH 7.5. The amount of specific proteins in each tube was determined by standard procedures described under MATERIALS AND METHODS.

peaks could be identified on either side of the adenylate kinase peak and corresponded to molecular weights of about 24,000 and 18,500. When the isozymes separated by electrofocusing were subjected to molecular sieving in the same column with the same marker proteins, relatively sharp, symmetrical peaks were observed and the molecular weight values of Table 1 were estimated. When the separated guanylate kinase isozymes from Sarcoma 180 cells were subjected to molecular sieving in the same column as employed in Fig. 5, similar molecular weight values were estimated, as shown in Table 1.

Kinetic Parameters of Guanylate Kinase Isozymes

The kinetic parameters of each isozyme were determined with ATP and GMP as the substrates (Table 2). In each case, the apparent Michaelis constant and V_{max} values were determined at a number of concentrations of the second substrate, and the data were replotted as described in the legend for Fig. 2 to estimate the true Michaelis constants. The kinetic parameters (Table 2) of the different isozymes in individual tissues do not differ strikingly, and the greatest differences seen are on the order of 2-fold. The Michaelis constants of the substrates dGMP and 8-azaGMP and the inhibition constants (K_i) for 6-thioGMP are shown in Table 3. The K_m values of Table 3 were not determined at infinite concentrations of the second substrate and therefore are apparent K_m values that may not be compared directly with the values of Table 2. Again it is of interest that all isozymes were inhibited by the analogue nucleotide, 6-thioGMP. The K_i values reported here are in excellent agreement with those reported earlier from this laboratory (2, 3), using less purified samples of guanylate kinase.

It was observed earlier that although inosine 5′-phosphate has no activity with guanylate kinase isolated from hog brain (1), there is weak but definite activity with guanylate kinase from human erythrocytes (3). Therefore the guanylate kinase isozymes separated by electrofocusing in Fig. 4b were examined for their reactivity with IMP. Because of the relatively low activity with IMP as the substrate and the limited amount of each isozyme available, a complete kinetic examination was not performed. However, with the erythrocytic isozymes of pI 4.9, 5.1, and 5.4, a reaction velocity was observed

TABLE 1

Isoelectric points, specific activities, and molecular weights of guanylate kinase isozymes

A purified sample of guanylate kinase from human erythrocytes (specific activity, 3.3 units/mg of protein) and Sarcoma 180 cells (specific activity, 1.1 units/mg protein) when electrofocused in a pH gradient from 4 to 6, four isozymes of erythrocytic guanylate kinase, and two isozymes from Sarcoma 180 cells were separated at their isoelectric points (Figs. 2b and 4b). The specific activity of the pooled fraction of each isozyme was determined after dialysis against 0.1 M Tris-acetate buffer, pH 7.5. The molecular weight of each isozyme was determined by molecular sieving, using a Bio-Gel P-100 column (1.0 × 60 cm). The samples added to the column contained the following in each 0.5 ml: about 0.2 unit of the isozyme, 1.5 mg of bovine plasma albumin, 1 μg of peroxidase, 1–2 units of adenylate kinase, and 2.5 mg of cytochrome *c*. Except for erythrocytic isozyme IV, the supply of which was sufficient for only one run, the molecular weight determinations were performed in duplicate and yielded almost identical values. The estimate of error takes into account the effect on the estimated molecular weight if the peak of activity had occurred in one tube on either side of the observed activity peak.

Isozyme	pI	Specific activity	Mol wt
		units/mg	
Erythrocytic			
I	4.9	0.91	21,000 ± 1,000
II	5.1	13.30	21,000 ± 1,500
III	5.4	17.80	18,500 ± 1,500
IV	5.8	0.58	24,000 ± 1,000
Sarcoma 180			
I	5.2	2.05	19,500 ± 500
II	5.5	9.40	20,000 ± 500

with IMP that was about 0.4% of the velocity with GMP as the substrate, while with the fourth isozyme (pI 5.8) the velocity was somewhat greater, about 1% of that seen with GMP as the substrate.

DISCUSSION

These investigations demonstrate, by the use of two electrophoretic techniques, the occurrence of four isozymes of guanylate kinase in human erythrocytes and two isozymes of guanylate kinase in Sarcoma 180 cells. Although the isoelectric points of the Sarcoma 180 guanylate kinase isozymes differ by about 0.3 pH unit, the isozymes resemble each other closely in molecular weight and kinetic behavior. On the other hand, the four human erythrocytic isozymes differ in molecular weight, varying from about 18,500 to 24,000. However, they do not differ strikingly in their behavior with various substrates. Interestingly, all six guanylate kinase isozymes are similar in their susceptibility to inhibition by 6-thioGMP, the greatest difference in K_i values being about 3-fold.

It has not yet been possible to carry out an extensive investigation of guanylate kinase isozymes in tumor tissues that are sensitive and resistant to 6-thioguanine. Such studies should be undertaken, since it is possible that certain instances of drug resistance to 6-thioguanine might be attributable to the presence of a guanylate kinase isozyme that is relatively resistant to inhibition by 6-thioGMP. There are still no generally accepted explanations of the mechanisms by which 6-thioguanine kills cells, or of its selectivity for certain tissues. We have found that guanylate kinases from normal hog brain (2), rat liver,[2] and human erythrocytes (3) and from the tumor Sarcoma 180 (2) are all inhibited to about the same extent by 6-thioGMP. Therefore, although inhibition of guanylate kinase may be a significant factor in the mechanism by which 6-thioguanine causes cellular death, the explanation of tissue selectivity must be sought elsewhere. Since it is established that 6-thioguanine must be converted to the nucleotide level to have cytolytic effects, it seems possible that other factors, such as differences from one tissue to the next in the hypoxanthine-guanine phosphoribosyltransferases, in rates of degradation of 6-thioguanine or 6-thioGMP, etc., might be involved in resistance to the cytolytic action of 6-thioguanine. For example, although the four human erythrocytic guanylate kinase isozymes are all inhibited competitively by 6-thioGMP, it has not been possible to demonstrate the accumulation of significant levels of 6-thioGMP in this cell. Therefore a current study in this laboratory involves examination of

[2] K. C. Agarwal and R. E. Parks, Jr., unpublished results.

TABLE 2

Kinetic parameters of guanylate kinase isozymes

The isozyme fractions used for the kinetic experiments were aliquots of pooled, dialyzed samples of the peaks in Figs. 2b and 4b. For each kinetic measurement, 1 ml of the reaction mixture contained 0.0038 unit of guanylate kinase isozyme, 100 μmoles of Tris-acetate (pH 7.5), 100 μmoles of KCl, 10 μmoles of $MgCl_2$, 1.5 μmoles of phosphoenolpyruvate, 1.25 micromolar units of pyruvate kinase, 2.5 micromolar units of lactate dehydrogenase, 0.15 μmole of NADH, and the substrates ATP and GMP at various concentrations. The oxidation of NADH was followed with time by the change in absorbance at 340 nm at 30°. True K_m and V_{max} values for each of the guanylate kinase isozymes were determined from replots of the reciprocals of apparent maximal velocities against the reciprocals of ATP or GMP concentrations. For each replot, four apparent V_{max} values were employed. Statistical analysis of each Lineweaver-Burk plot was performed using Cleland's computer program (20), and most of the apparent V_{max} values utilized in the replots had standard errors substantially lower than ±10%, in many cases being on the order of ±1–2%.

Isozyme	ATP		GMP	
	K_m	V_{max}	K_m	V_{max}
	$M \times 10^4$	$-A_{340}\ min^{-1}$	$M \times 10^5$	$-A_{340}\ min^{-1}$
Erythrocytic				
I	1.09	0.050	1.25	0.048
II	0.83	0.057	1.85	0.069
III	1.35	0.059	2.04	0.062
IV	1.61	0.065	2.00	0.062
Sarcoma 180				
I	1.00	0.082	1.49	0.048
II	0.68	0.048	1.56	0.048

TABLE 3

Kinetic parameters of guanylate kinase isozymes from human erythrocytes and Sarcoma 180 ascites cells with dGMP, 8-azaGMP, and 6-thioGMP

The reaction constituents were similar to those described in Table 2, except that 4.0 mM ATP was used throughout. The K_m values for dGMP and 8-azaGMP were obtained from Lineweaver-Burk plots. The K_i values of 6-thioGMP were determined by replotting the slope values obtained at three different concentrations of 6-thioGMP against the concentration of 6-thioGMP (2). In each case classical patterns for competitive inhibition between GMP and 6-thioGMP were seen.

Isozyme	K_m		K_i
	dGMP	8-AzaGMP	6-ThioGMP
	M	M	M
Erythrocytic			
I	5.9×10^{-5}	1.1×10^{-4}	7.5×10^{-5}
II	5.9×10^{-5}	0.9×10^{-4}	1.3×10^{-4}
III	5.9×10^{-5}	1.1×10^{-4}	2.1×10^{-4}
IV	3.3×10^{-5}	1.0×10^{-4}	7.5×10^{-5}
Sarcoma 180			
I	2.5×10^{-5}	3.8×10^{-5}	1.7×10^{-4}
II	1.4×10^{-5}	3.0×10^{-5}	6.7×10^{-5}

the comparative substrate activities of guanine, hypoxanthine, and various purine antimetabolites with hypoxanthine-guanine phosphoribosyltransferases from various tissues, including the human erythrocyte.

The occurrence of multiple isozymes of guanylate kinase in human erythrocytes raises the question of genetic variation, especially since the enzyme source in most of these studies was pooled blood from about 100 different individuals. When the erythrocytic guanylate kinase isozymes from two individuals were examined by the agarose electrophoresis overlay method, however, the three major guanylate kinase isozymes were clearly detected in each case. A much more extensive study of these isozymes in a large number of individuals will be necessary before conclusions can be drawn about genetic implications. In any case, a physiological explanation of the occurrence of these different isozymes is not presently apparent.

A phenomenon detected in the course of isolation of the erythrocytic isozymes was the apparent occurrence of aggregation. When molecular weights were determined at an early stage of purification by the molecular sieving method of Andrews (19), a broad

peak of guanylate kinase activity, varying from about mol wt 40,000 to 20,000, was seen. At the highest stages of purification, after resolution of the isozymes, sharp peaks of activity were observed. Since the individual erythrocytic guanylate kinase peaks varied in molecular weight from 18,000 to about 24,000, it is obvious that an aggregation phenomenon is not the explanation for the occurrence of the multiple electrophoretic bands.

One may not safely conclude that different species of an enzyme exist simply because several bands of activity are observed by electrophoretic techniques. The possibility exists that the same protein is present in two or more interconvertible forms. However, we have not seen any evidence of interconvertibility with guanylate kinase from human erythrocytes and Sarcoma 180 ascites cells. It will be necessary to wait for further purification and characterization of the individual bands of enzymatic activity to draw definitive conclusions on the existence of isozymes.

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