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DEOXYGUANOSINE KINASE OF NEONATAL MOUSE SKIN TISSUE

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Summary

Deoxyguanosine kinase (ATP: deoxyguanosine 5'-phosphotransferase) activity has been identified in neonatal mouse skin tissue. This activity which has a molecular weight of 44 000 was shown to be highly specific for deoxyguanosine as a substrate. Unique to deoxynucleoside kinases was the observation that this enzyme possessed a pH optimum of 5.2. In dilute solutions catalytic activity is lost, however the enzymatic activity can be stabilized by the addition of 20 μM MgATP or ATP. Kinetic analysis gave an apparent K_m for deoxyguanosine of 7 μM. Double-reciprocal plots of activity vs. MgATP concentration produced a broken line with the break occurring at 0.5 mM MgATP. Below this concentration an apparent K_m for MgATP of 23 μM was measured; above 0.5 mM MgATP an apparent K_m of 625 μM was calculated. Mouse skin deoxyguanosine kinase was strongly inhibited by dGTP, dGDP and UDP. dGTP was a competitive inhibitor of deoxyguanosine with an apparent K_i of 1.9 μM. UDP ($K_{i,app}$ = 3 μM), dGDP ($K_{i,app}$ = 0.7 μM) and dGTP ($K_{i,app}$ = 0.07 μM) were competitive inhibitors of MgATP when the concentration of MgATP was greater than 0.5 mM.

Introduction

Deoxyguanosine kinase (ATP: deoxyguanosine 5'-phosphotransferase, EC 2.7.4.8) catalyzes the first reaction in the reutilization of deoxyguanosine for the biosynthesis of dGTP, one of the four precursors for DNA synthesis. It is generally understood that this type of salvage reaction is advantageous to the cell, in that the overall consumption of energy to synthesize dGTP by this

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route is much less than that required by the de novo pathway. Deoxyguanosine is metabolically situated in a pivotal position between pathways leading to its degradation, which is initiated by the enzyme purine nucleoside phosphorylase [1], and its reutilization, a sequence of reactions beginning with deoxyguanosine kinase [2,3]. The metabolic controls which regulate the subcellular decision to either anabolically or catabolically metabolize the deoxynucleoside are not known, however some data have been reported which suggest that deoxyguanosine kinase may play an important role as a regulatory protein [3].

Deoxyguanosine kinase has been detected in several mammalian systems, but detailed studies are rare. A deoxycytidine kinase which also exhibits deoxyguanosine substrate specificity, however to a much less extent, has been purified to homogeneity from calf thymus [4] but no other pure protein has been reported to possess the ability to phosphorylate deoxyguanosine. Gower et al. [3] have shown, in rat liver and calf thymus, that two deoxyguanosine kinase activities occur within the cell, one in mitochondria and the other in the cytosol. The cytosolic form appeared to be the above-mentioned deoxycytidine kinase, whereas the mitochondrial form had much greater specificity toward deoxyguanosine.

Recent studies by Green and Lewis [2] reported a partially purified deoxyguanosine kinase from pig skin which was highly specific for deoxyguanosine as substrate. This enzyme was shown to be competitively inhibited by its end product dGTP. In contrast to the present study was the observation that pig skin deoxyguanosine kinase exhibited a pH optimum of 8.0—8.5, although activity was high over a broad range of pH (5.5—9.0).

This report on deoxyguanosine kinase from newborn mouse skin tissue was prompted by the observation that its catalytic activity showed a sharply acidic pH optimum. This and other characteristics suggest that the neonatal mouse skin activity is distinctly different from the pig skin enzyme as well as from other reported deoxyguanosine kinase activities. Included in this study is a characterization of the catalytic properties of mouse skin deoxyguanosine kinase and an investigation of the inhibition of the kinase by metabolic nucleotides, two of which (UDP and dGTP) may play an important role in the physiological control of deoxyguanosine kinase activity.

Materials and Methods

Materials. Deoxyadenosine and deoxyguanosine were from Calbiochem. Other nucleosides and nucleotides used for this study were Sigma-grade reagents and all other chemicals were reagent grade. [8-^{3}H]Deoxyguanosine (1.4—26.4 Ci/mmol), [8-^{3}H]deoxyadenosine (14.4 Ci/mmol), [5-^{3}H]deoxycytidine (5.9 Ci/mmol), [^{3}H-*methyl*]thymidine (65 Ci/mmol), [2,8-^{3}H]adenosine (60.5 Ci/mmol), [8-^{3}H]guanosine (19.7 Ci/mmol), [5-^{3}H]cytidine (28 Ci/mmol), [5-^{3}H]uridine (25.4 Ci/mmol) and [2,8-^{3}H]ATP (41.6 Ci/mmol) were all from ICN. PEI-cellulose was from Brinkman Instruments. The neonatal mice were obtained from pregnant Swiss-Websters (Simonsen Labs, Gilroy, CA).

Deoxyguanosine kinase assay. Deoxyguanosine kinase activity was assayed in a total reaction volume of 125 μl, which contained 0.1 M tris-maleate, pH 5.2 measured at 37°C/1 mM dithiothreitol/10 mM ATP/10 mM $MgCl_2$/140 μM

deoxyguanosine (2—5 μCi)/25 μl enzyme. 1 unit of enzyme activity is defined as the amount of protein required to convert 1 nmol deoxyguanosine to 5′-dGMP, in 30 min at 37° C. The amount of enzyme assayed was between 0.1 and 0.5 units, and the rate of product formation was always constant over the period of incubation. Assay were carried out as previously reported [2].

For all calculations of kinetic constants, the following dynamic equilibria for ATP and Mg^{2+} were assumed to be functional:

$$\begin{array}{ccc} H^+ + MgATP^{2-} & \overset{(a)}{\rightleftharpoons} & MgATP^- \\ \downarrow\uparrow (d) & & \uparrow\downarrow (b) \\ Mg^{2+} + ATP^{4-} + H^+ & \overset{(c)}{\rightleftharpoons} & ATP^{3-} + Mg^{2+} \end{array}$$

The proportion of added ATP and Mg^{2+} which actually exists as the complex between the two depends on concentration and pH. Values for the constants of these equilibria were obtained from the work of Phillips et al. [5]. For equilibria (a) and (c), the pK values were taken to be 5.21 and 7.04, respectively. For equilibria (b) and (d), K_{dis} was calculated as $1.86 \cdot 10^{-3}$ and $2.82 \cdot 10^{-5}$, respectively. All the values of the MgATP concentrations used for the derivation of kinetic constants come from this calculation and plots of kinetic data were drawn in accordance with a least squares fit of the data given by linear regression analysis.

Product identification. The reaction was terminated by the addition of dimethylamide, to which was added bovine serum albumin as a carrier. Following centrifugation of this mixture, an aliquot of the supernatant was mixed with authentic standards of dAMP, dIMP, dGMP and XMP and injected onto a Partisil 10-SAX column (250 mm × 4 mm) equilibrated with 7 mM KH_2PO_4, pH 4.0 measured at 25° C. Eluted samples were assayed for radioactivity and the positions of the peaks of radioactivity were then compared to the retention times of the standards.

To measure the extent of deoxyguanosine di- and triphosphate formation, the column was equilibrated with 0.01 M $NH_4H_2PO_4$, pH 2.8 measured at 25° C. After injection of reaction product, a linear gradient was initiated starting with the equilibration buffer and going to 0.5 M $NH_4H_2PO_4$, pH 4.8 measured at 25° C, according to the method of Pross et al. [6]. Fractions were collected and quantitated for radioactivity and the peaks of radioactivity were compared to the retention times of the standards. Using this technique all of the radioactive product was accounted for as dGMP (85%), dGDP (2%) and dGTP (13%).

Determination of nucleotidase (or phosphatase) activities. To a 1.0 ml vol. of assay solution was added 0.25 ml of the enzyme preparation to be tested. The total reaction volume of 1.25 ml contained 0.1 M Tris-maleate, pH 5.2 measured at 37° C/1 mM dithiothreitol/1 mM monophosphate nucleotide. Incubation was for 30 min at 37° C. The reaction was stopped by immersing the tubes in a boiling water bath for 3 min. All the tubes were then frozen, thawed, and centrifuged, and 0.5 ml of the supernatant taken for determination of inorganic phosphate according to the method of Eibl and Lands [7].

Determination of deoxyguanosine phosphorylase activity. The enzyme preparation to be tested was assayed in the usual way with a standard assay

solution that included 4 μCi [8-^{3}H]deoxyguanosine. 10 μl supernatant from each assay tube were spotted on top of standards on Whatman No. 1 paper. Descending chromatography using 0.5 N NH_3 and 0.05 M EDTA as solvent separated guanine, deoxyguanosine, and dGMP according to the method of Uitendaal et al. [8]. Following chromatography, the spots representing these standards were cut out and quantitated for radioactivity.

Determination of ATPase activity. The assay solution contained 0.1 M Tris-maleate, pH 5.2 measured at 37°C/1 mM dithiothreitol/10 mM $MgCl_2$/10 mM [^{3}H]ATP (6 μCi/2.5 μmol)/0.025 ml enzyme in a final volume of 0.125 ml. After incubation for 30 min at 37°C, the reaction was stopped with 0.125 ml methanol, frozen, thawed and centrifuged. 10 μl supernatant were then spotted on PEI-cellulose and cochromatographed with AMP, ADP, ATP or adenosine as standards, using 0.85 M KH_2PO_4, pH 3.4 measured at 25°C, as solvent [9]. Following chromatography, the areas where the standards migrated were cut out and quantitated for radioactivity.

Partial purification of deoxyguanosine kinase activity from neonatal mouse skin. 3-day-old mice were killed by decapitation and their skins removed. The skins were weighed and stored at −20°C until needed. A typical isolation began with 30 g tissue and followed the method of Green and Lewis [2], with some modifications. All steps in the procedure were done at 4°C. The frozen skins were washed twice with homogenizing buffer, 0.1 M Tris-acetate, pH 8.0 measured at 25°C/25 mM mercaptoethanol, and minced with scissors. The minced material was homogenized with a ground glass homogenizer in 60 ml buffer. The homogenate was centrifuged for 15 min at 27 000 × *g*, and the pH of the resulting supernatant (52 ml) was lowered to 5.7 by the dropwise addition of 0.5 M acetic acid. This material was centrifuged for 12 min at 27 000 × *g* and the pH of the supernatant was adjusted to 8.0 (4°C) by the dropwise addition of 2 M NaOH. To 49 ml of this material was added 4.9 ml 10% streptomycin sulfate (w/v) dissolved in homogenizing buffer. 40 min were allowed for equilibration, followed by centrifugation for 15 min at 27 000 × *g*. To the supernatant (52 ml), 12.6 g $(NH_4)_2SO_4$ was added slowly and with stirring. After a 1-h equilibration the sample was centrifuged. To the supernatant of 56 ml was added 3.5 g ground $(NH_4)_2SO_4$ followed by equilibration (1 h); the insoluble material constituted the 40—50% $(NH_4)_2SO_4$ fraction. Centrifugation resulted in a supernatant of 57 ml to which was added, with stirring, 3.7 g $(NH_4)_2SO_4$, followed by 1 h of equilibration. This precipitate constituted the 50—60% $(NH_4)_2SO_4$ fraction. The insoluble material from each of these fractions was dissolved in homogenizing buffer, dialyzed against two changes of the same buffer and stored at −20°C. Stored in this way, the deoxyguanosine kinase activity was stable for at least 4 months. Protein concentration of aliquots of the various fractions was monitored using the Lowry determination according to the method of Sutherland et al. [10], using bovine serum albumin as a standard.

Results

Partial purification. Table I is a summary tabulation of a typical fractionation of deoxyguanosine kinase activity, starting with 30 g neonatal mouse skin.

TABLE I

PARTIAL PURIFICATION OF DEOXYGUANOSINE KINASE FROM NEONATAL MOUSE SKIN

Activity with deoxyguanosine as substrate was measured at pH 5.2 (37°C). [deoxyguanosine] = 140 μM; [ATP] = 10 mM and [$MgCl_2$] = 10 mM. 1 unit of activity is that amount of protein which catalyzes the conversion of 1 nmol substrate to product in 30 min.

Fraction	Volume (ml)	Protein (mg/ml)	Total units	Specific activity (units/mg)	Recovery (%)	Fold purification
I. Homogenate supernatant	52	18.9	1456	1.48	100	1
II. pH	50	10.2	1250	2.45	86	1.7
III. Streptomycin Sulfate	53	11.0	1283	2.20	88	1.5
IV. $(NH_4)_2SO_4$ (40—50%)	4	9.8	824	21.0	57	14

Most of the deoxyguanosine kinase activity was consistently salted out in the 40—50% saturated fraction (Fraction IV), and in spite of the fact that the degree of purification was low, this procedure did result in the fractionation of a protein which was relatively free of other deoxynucleoside kinases. Thymidine kinase was found only in the 0—40% $(NH_4)_2SO_4$ fraction (assayed at pH 8.0). When deoxycytidine kinase was assayed at pH 8.0, activity was measured in both the 40—50% and 50—60% fractions, however at levels approx. 10-times lower than the other deoxynucleoside kinases. The 40—50% and 50—60% fractions also possessed an activity(ies) which converted deoxyadenosine into dIMP when assayed at pH 6.2. It is shown below that when enzyme assays were conducted, employing conditions which favored deoxyguanosine kinase activity (pH 5.2), that the only contaminating activity measured was when deoxyadenosine was added as substrate, and this only to a minor extent.

Tests for auxiliary reactions. Measurement of reaction rates could be affected by enzymes other than deoxyguanosine kinase which may be present in the enzyme preparation and which act on the same substrates and products. Two activities which would reduce substrate availability are purine nucleoside phosphorylase and ATPase. When assayed according to the method of Uitendaal et al. [8], purine nucleoside phosphorylase consumed less than 5% of the substrate in an incubation time of 30 min. With regard to ATPase only 2% of the ATP was hydrolyzed during a 30-min incubation by an amount of enzyme comparable to that used for a typical assay when ATP concentration = 10 mM.

Enzymes which, if present, would directly affect the amount of product measured are 5′-nucleotidase and phosphatase. With 1 mM nucleotide substrate and using a comparable amount of enzyme as would be used in a typical assay, about 0.5% of the dGMP was hydrolyzed to deoxyguanosine in 30 min of incubation.

Therefore, assays of deoxyguanosine kinase activity were not significantly altered by reactions competing for the same substrates. Furthermore, in all studies, reaction rates for deoxyguanosine kinase were measured to be linear with respect to time and directly proportional to protein concentration under the reaction conditions used.

pH optima. Fig. 1 shows that deoxyguanosine kinase activity in newborn

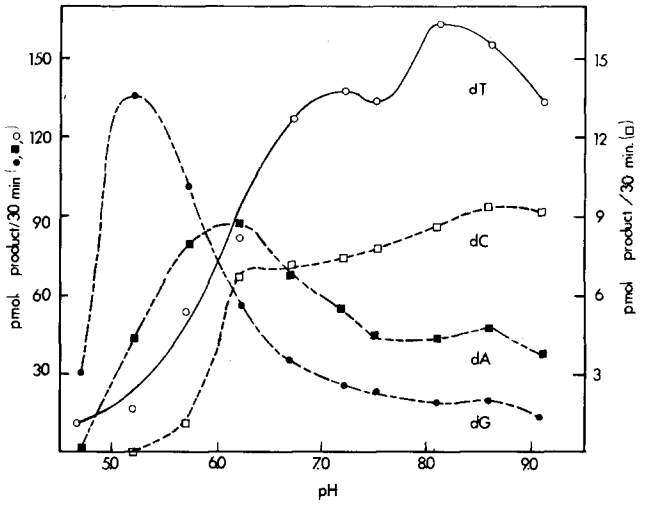

Fig. 1. Effect of pH on deoxynucleoside kinase activities. The assay of the different activities followed the same basic procedure as given in Materials and Methods for the assay of deoxyguanosine kinase. [ATP] = 10 mM and [$MgCl_2$] = 10 mM in all cases. In each case, deoxynucleoside concentration equals 50 μM. dT, deoxythymidine; dC, deoxycytidine; dA, deoxyadenosine and dG, deoxyguanosine.

mouse skin had an acidic pH optimum. The pH optima of each of the deoxynucleosides tested were as follows: 5.2 for deoxyguanosine, 6.2 deoxyadenosine (or deoxyinosine), 8.6 for deoxycytidine and 8.1 for thymidine. These activities came from different $(NH_4)_2SO_4$ fractions: deoxyguanosine kinase, 40—50%; deoxyadenosine (deoxyinosine) and deoxycytidine kinases, 55—65% and thymidine kinase, 0—40%.

When Tris-acetate was substituted for Tris-maleate in the neutral and basic range of the pH profile, no change in the shape of the curve or in the catalytic activity of deoxyguanosine kinase was observed.

Cation requirement. When chloride salts of divalent cations were tested as substitutes for Mg^{2+}, only Zn^{2+}, Mn^{2+}, Ca^{2+} and Cd^{2+} were shown to be effective. In the absence of divalent cation, 8% of the activity of the kinase was measured.

Phosphate donor specificity. ATP was the most effective of the various nucleoside triphosphates as a phosphate donor for the deoxyguanosine kinase activity (Table II). In terms of purine nucleoside triphosphates, the phosphate donor requirement was such that it did not tolerate deviations from the adenine base nor the ribose sugar. As for the pyrimidine nucleoside triphosphates, CTP was significantly preferred over UTP and dTTP appears to be the most effective substitute for ATP.

Phosphate acceptor specificity. When deoxyguanosine, deoxyadenosine, deoxycytidine, deoxythymidine, adenosine, uridine and cytosine were tested for phosphate acceptor activity ([acceptors] = 140 μM) only deoxyguanosine and deoxyadenosine were active. Deoxyadenosine (deoxyinosine) was phosphorylated to about 20% of the level of deoxyguanosine. It is important to note that when deoxyadenosine was the substrate and using the same procedure as was employed for product identification of deoxyguanosine kinase, all

TABLE II

PHOSPHATE DONOR SPECIFICITY FOR DEOXYGUANOSINE KINASE

Fraction IV was assayed for deoxyguanosine kinase activity in the standard way except that the various nucleoside triphosphates were substituted for ATP in combination with Mg^{2+}. The [cation]/[ATP] = 1. [deoxyguanosine] = 140 μM. [triphosphate] = 10 mM and [cation] = 10 mM.

Nucleoside triphosphate	% of activity	Nucleoside triphosphate	% of activity
ATP	100	dATP	6
GTP	9	dGTP	0
CTP	67	dCTP	35
UTP	20	dTTP	84

the product was identified as dIMP. There are two metabolic pathways which could account for dIMP formation. One would be deamination of deoxyadenosine followed by phosphorylation; the other is phosphorylation of deoxyadenosine followed by deamination. Preliminary results indicated that more than 50% of the deoxyadenosine was being deaminated during a 30-min incubation; therefore, the first-mentioned pathway probably accounts for dIMP formation by Fraction IV.

That deoxyguanosine is the preferred substrate was supported by inhibition studies. When deoxyadenosine (70 μM) was tested as an inhibitor of deoxyguanosine (14 μM) as substrate, 9% inhibition was measured. Using deoxyinosine (70 μM) as an inhibitor caused only 17% inhibition of deoxyguanosine kinase activity.

Stability of deoxyguanosine kinase activity. When deoxyguanosine kinase was assayed as a function of protein concentration, it was seen that the activity was linear at concentrations greater than 80 μg protein/ml of assay solution. As Fig. 2 shows, at less than this concentration, the activity dropped off hyperbolically. Fig. 2 also shows the effect of adding 12.5 mM ATP and 12.5 mM

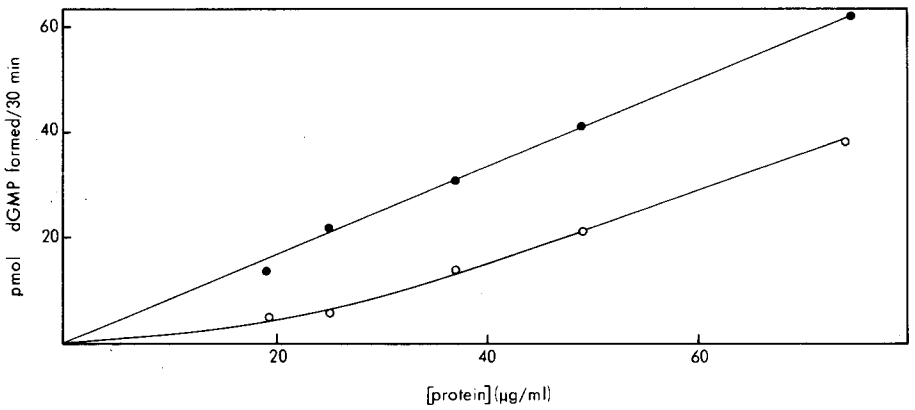

Fig. 2. Stabilization to dilution of deoxyguanosine kinase by ATP. Fraction IV was diluted with 0.1 M Tris-maleate/1 mM dithiothreitol, pH 5.2 (37°C), or with the same buffer supplemented with 12.5 mM ATP and 12.5 mM $MgCl_2$, and assayed immediately. Protein concentration is given for the final assay volume of 0.125 ml. [deoxyguanosine] = 70 μM; [ATP] = 12.5 mM and [$MgCl_2$] = 12.5 mM. ●———●, +ATP-Mg; ○———○, —ATP-Mg.

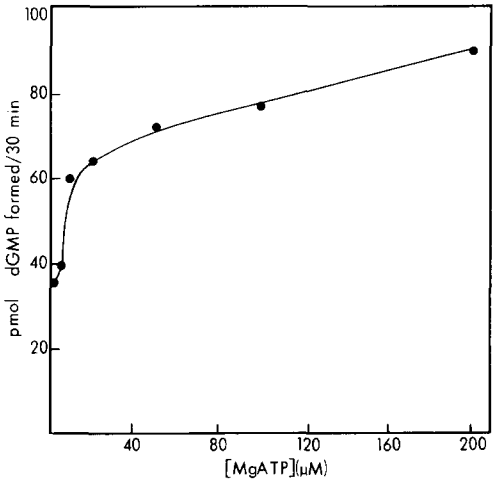

Fig. 3. Effect of MgATP concentration on the stability of deoxyguanosine kinase. Fraction IV was diluted to 0.25 mg/ml with 0.1 M Tris-maleate/1 mM dithiothreitol, pH 5.2 (37°C), supplemented with equimolar amounts of ATP and $MgCl_2$ and assayed immediately. The final assay solution contained [deoxyguanosine] at 140 μM, [ATP] at 10 mM and [$MgCl_2$] at 10 mM.

$MgCl_2$ to the buffer in which the enzyme was diluted. This stabilization was titrated (Fig. 3) and it can be seen that an added concentration of ATP and $MgCl_2$, as low as 20 μM, considerably enhanced the activity of the diluted enzyme. An equivalent stabilization was seen with ATP alone, but Mg^{2+} alone was ineffective. When the four common deoxyribo- and ribonucleoside triphosphates were tested for their ability to stabilize deoxyguanosine kinase activity, none were as effective as ATP.

Kinetic studies. An apparent K_m value for deoxyguanosine of 7 μM was determined from a double-reciprocal plot (Fig. 4). The concentration of deoxyguanosine was varied from 4.9 to 56 μM while the ATP concentration was held constant at 7.4 mM (additions of 10 mM ATP and 10 mM $MgCl_2$ to the assay solution). In separate experiments (data not shown), replotting the intercepts of the family of straight lines, generated from double-reciprocal plots vs. various substrate concentrations, confirmed the K_m value for deoxyguanosine. The family of lines were generated from assays in which concentrations of deoxyguanosine varied from 5.2 to 6.3 μM and MgATP ranged from 21 to 390 μM.

For the determination of an apparent K_m value for MgATP the concentrations were varied from 14 μM to 2.5 mM and deoxyguanosine concentration was held constant at 280 μM. The double-reciprocal plot of these data exhibited a broken line (Fig. 5A) with the break occurring at approx. 0.5 mM MgATP. That the break is significant is indicated by the fact that the correlation coefficients for the two lines to either side of the break are 0.9562 and 0.9999, whereas the correlation coefficient for the least-squares fit drawn through all the points is 0.8092. If the broken line is treated as two straight lines, the following results are calculated from an Eadie-Scatchard plot. From the line representing MgATP less than 0.5 mM, an apparent K_m of 23 μM for MgATP was calculated. For the line representing [MgATP] greater than 0.5

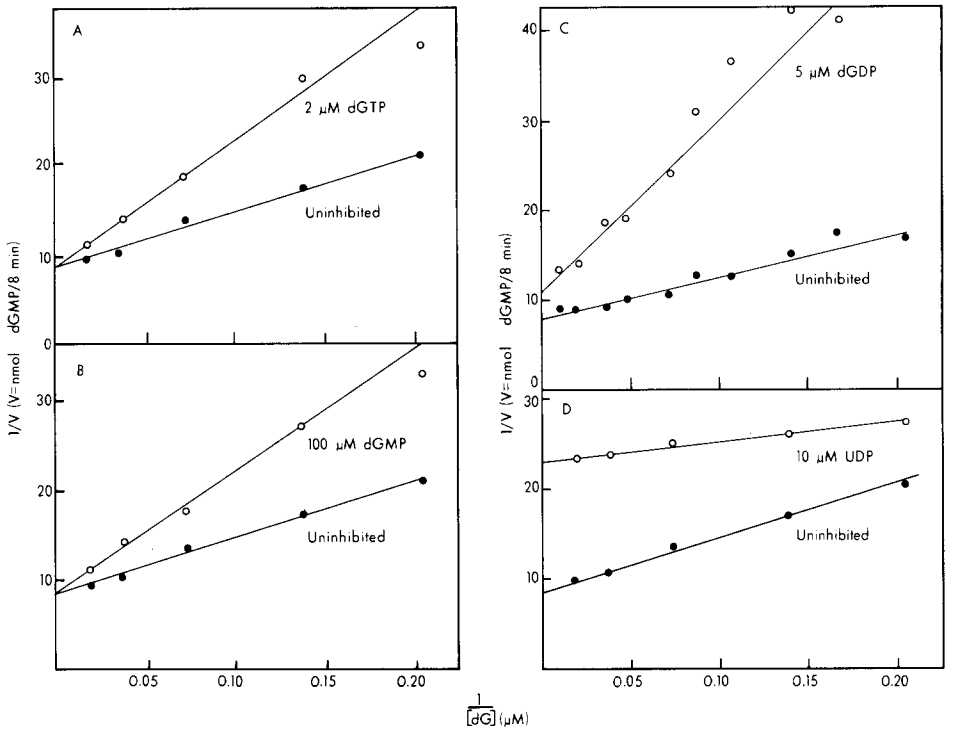

Fig. 4. Effects of nucleotides on deoxyguanosine kinase activity at various concentrations of deoxyguanosine. [deoxyguanosine] was varied from 4.9 μM to 56 μM, and [MgATP] was held constant at 7.4 mM (10 mM added substrate). Incubation was for 8 min at 37° C.

mM, the constant is 625 μM. The possibility that the break in the line in Fig. 5A was caused by the high [ATP] to [MgATP] ratio which exists below 0.5 mM MgATP was tested by maintaining a constant added excess of 5 mM $MgCl_2$ (Fig. 5B). This addition favors the formation of MgATP, so that the complex was the predominant species at any MgATP concentration. Under these conditions the break in the curve still occurred in the same position with respect to MgATP concentration as it did without the excess $MgCl_2$. Linear regression analysis gave a correlation coefficient of 0.9943 for data points representing MgATP concentrations less than 0.5 mM. For those above this concentration, a correlation coefficient of 0.9958 was obtained. An apparent K_m of 0.41 mM was derived from the data points representing MgATP concentration less than 0.5 mM; a K_m of 2.0 mM was measured for MgATP concentration above 0.5 mM.

General inhibition studies. To determine which nucleotides are involved in regulating deoxyguanosine kinase activity, screening studies were undertaken in which 10 mM nucleotide were added to the standard reaction solution. The most inhibitory nucleotides were selected and tested against a low deoxyguanosine concentration (which approximated the K_m value for this substrate) and a saturating concentration of MgATP, and conversely against a nearly-saturating concentration of deoxyguanosine and low concentration of MgATP (Table III). When deoxyguanosine concentration approximated its K_m value, only dGMP,

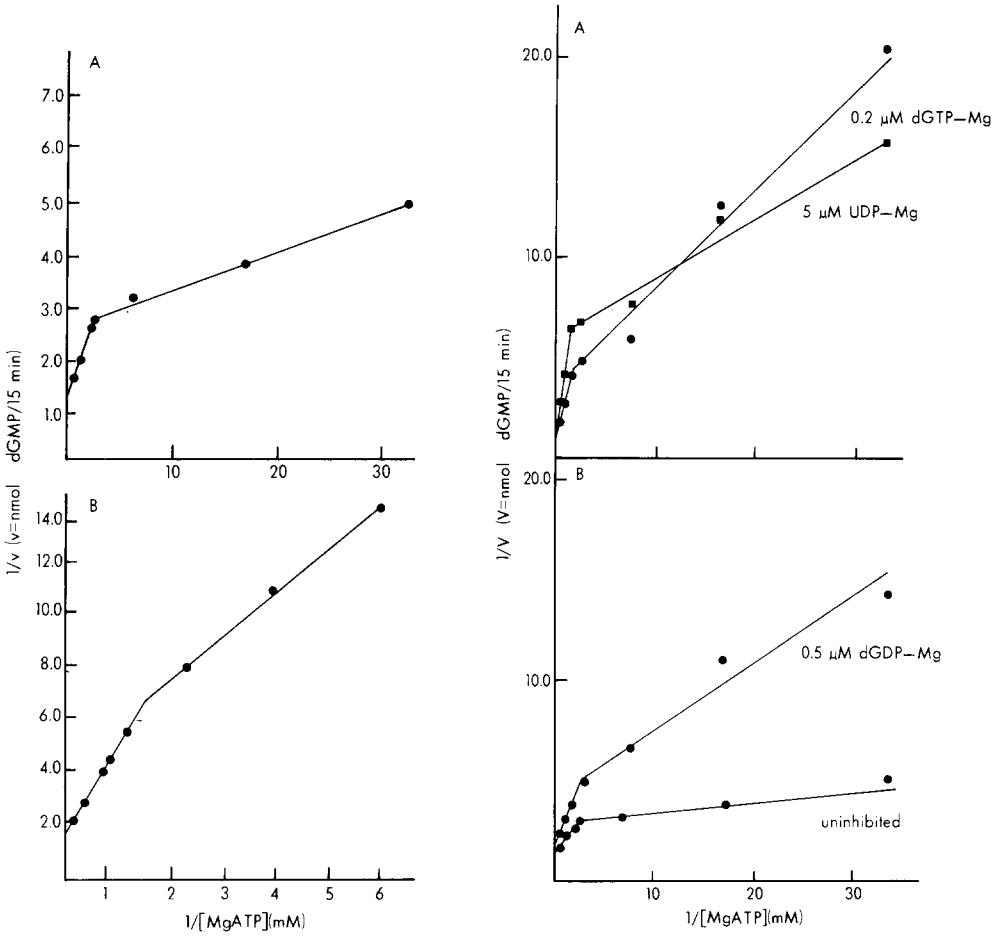

Fig. 5. Deoxyguanosine kinase activity at various concentrations of MgATP. A. Fraction IV was assayed for deoxyguanosine kinase activity with [deoxyguanosine] held constant at 280 μM and equimolar concentrations of ATP and $MgCl_2$ varied from 0.13 to 4 mM. Under these conditions, [MgATP] varied from 14 μM to 2.5 mM. Incubation was for 15 min at 37° C. B. Prior to assay, Fraction IV was dialyzed against 0.1 M sodium acetate/25 mM mercaptoethanol, pH 8.0, 25° C. It was then assayed for deoxyguanosine kinase in the standard way except that 0.1 M sodium acetate replaced 0.1 M Tris-maleate as the buffering species in the assay solutions. [deoxyguanosine] was held constant at 280 μM and 5 mM $MgCl_2$ was added to equimolar concentrations of added ATP and $MgCl_2$, which varied from 0.2 to 6.0 mM. Under these conditions, [MgATP] varied from 0.17 to 5.2 mM. Incubation was for 15 min at 37° C.

Fig. 6. Effects of nucleotides on deoxyguanosine kinase activity at various concentrations of MgATP. Equimolar concentrations of added ATP and $MgCl_2$ varied from 0.13 to 4.0 mM. At these concentrations, [MgATP] varied from 14 μM to 2.5 mM. [deoxyguanosine] was held constant at 280 μM. Incubation was for 15 min at 37° C.

dGDP, UDP and dGTP were inhibitory; similarly, when MgATP concentration was lowered to 9 μM, dGMP, dGDP, dTDP, UDP and dGTP were markedly inhibitory at the concentrations tested.

Kinetic analysis of inhibitors. As Fig. 4 shows, dGMP and dGTP appear to be competitive inhibitors with deoxyguanosine as substrate. K_i values of 180 μM and 1.9 μM were calculated for dGMP and dGTP, respectively. This same figure

TABLE III

EFFECT OF NUCLEOTIDES

Fraction IV was assayed for deoxyguanosine kinase activity in the standard way. The assay solution was supplemented with the inhibitors below at the indicated concentrations. A: [deoxyguanosine] = 14 μM, [MgATP] = 7.4 mM; B: [deoxyguanosine] = 140 μM, [MgATP] = 9 μM.

Nucleotide inhibitor	% activity			
	A		B	
	10 μM	100 μM	10 μM	100 μM
dGMP	98	48	83	28
dADP	98	90	87	43
dGDP	21	3	0	0
dTDP	102	79	57	35
GDP	97	83	56	31
UDP	51	22	23	13
dATP	103	80	52	26
dGTP	10	1	0	0
GTP	100	84	45	23
UTP	103	84	109	122

also shows the kinetics of inhibition given by dGDP and UDP. Each of these inhibitors exhibit both a K_m and V effect on catalytic activity. The pattern of inhibition given by UDP is unusual in that the inhibited line diverges away from the control as the deoxyguanosine concentration becomes larger. Fig. 6 shows the patterns of inhibition given by UDP, dGDP and dGTP when MgATP concentrations were varied while holding deoxyguanosine concentration at a saturating level. With all three of these compounds, the mechanism of inhibition appears to be the same. The lines produced by the reciprocal plots of the uninhibited and inhibited reactions all break at the same point. Furthermore, as MgATP concentrations exceeded 0.5 mM, the inhibition of each of the three nucleotides becomes competitive with respect to MgATP. For this range of substrate concentrations, K_i values were calculated to be 3 μM, 0.7 μM and 0.07 μM for UDP, dGDP and dGTP, respectively.

Molecular weight studies. An estimate of the molecular weight of deoxyguanosine kinase was undertaken via glycerol gradient centrifugation. A molecular weight determination was of interest since it was possible that the break in the Lineweaver-Burk plot, where MgATP concentration was varied from 14 μM, to 2.5 mM was mediated by a change in molecular weight. The enzyme was exposed to either no MgATP or 1.7 mM MgATP values representing MgATP concentrations on either side of the break point (0.5 mM MgATP). A molecular weight of 44 000 was determined for catalytic activity when the enzyme was sedimented at pH 7.8, in the absence of MgATP. No difference in the molecular weight was detected in an MgATP treated sample.

Discussion

Novel to this study is the observation that mouse skin deoxyguanosine kinase has a distinctly acidic pH optimum. Investigations of deoxyguanosine

kinase from other sources showed that those activities were optimally active over a broad range of pH including both basic and acidic values [2,11]. Furthermore, mouse skin deoxyguanosine kinase with its optimium at pH 5.2 is characteristically dissimilar to each of the other three deoxynucleoside kinases of mouse skin tissue.

Even though the fractionation of the enzymatic activity was limited, the results of three experiments suggest the activity in this report is a specific deoxyguanosine kinase and that the enzyme preparation used in this study (Fraction IV) is not significantly contaminated with other deoxynucleoside kinase activities. The first comes from $(NH_4)_2SO_4$ fractionation data in which deoxyguanosine kinase activity was concentrated in the 40—50% fraction, (Fraction IV), whereas deoxycytidine and deoxyadenosine (deoxyinosine) kinases predominated in the 50—60% fraction and thymidine kinase was found in the 0—40% fraction. Secondly, when Fraction IV was assayed with an equal concentration of either deoxyguanosine or deoxyadenosine as substrate, 5-times as much product was formed with deoxyguanosine. Finally, when deoxyguanosine was employed as substrate in the presence of a 5-fold excess of deoxyadenosine, the deoxyguanosine kinase activity was inhibited by only 9% and a 5-fold excess of deoxyinosine caused only a 17% inhibition of deoxyguanosine kinase activity. No activity was measured with ribonucleosides as substrates.

A comparison of neonatal pig [2] and mouse skin deoxyguanosine kinase reveals several unexpected differences between these catalytic activities. Already mentioned is the acidic pH optimum of the mouse enzyme (mouse, pH 5.2; pig, pH 8—8.5). The apparent K_m for MgATP of 3.3 mM for pig skin is significantly larger than that measured for mouse tissue. Furthermore, normal Michaelis-Menten kinetics were measured for the pig enzyme for MgATP as a substrate. The apparent K_m value for deoxyguanosine as substrate is lower for the pig than for mouse enzyme (pig, 0.31 μM; mouse, 7 μM). Other differences were reflected in the influence upon catalytic activity by metal ion requirements and in the molecular weight of the two activities (pig = 58 500; mouse = 44 000). It is not known if these differences are a reflection of the heterogeneous nature of the enzyme preparations employed or if they are related to species specific enzymes.

It has been shown by Gower et al. [3] that two catalytic activities for deoxyguanosine kinase exist in mammals. One is found in the cytosol, is associated with a predominate deoxycytidine substrate specificity, exhibits an ability to phosphorylate deoxyadenosine and has a pH optimum near 8.0. The other catalytic activity found in mammals appears to be located in the mitochondria. Preliminary studies with neonatal mouse skin mitochondria suggest that catalytic activity isolated from this organelle exhibits deoxyguanosine specificity at pH 5.2 and negligible activity at pH 8.0.

That a broken line is characteristic of the double-reciprocal plot of a deoxynucleoside kinase activity is not unique to this study. Ives and Durham observed such a transition with deoxycytidine kinase from calf thymus [12]. With deoxyguanosine kinase the break in the curve does not appear to be related to equilibrium ratios of [ATP] and [MgATP]. This was shown by shifting the ratio of [ATP]/[MgATP] by adding excess $MgCl_2$ to the reaction solutions.

This addition has the effect of increasing the $[Mg^{2+}]/[MgATP]$ and reducing the [ATP]/[MgATP]. Without added $MgCl_2$ and at concentrations less than 0.5 mM MgATP, this latter ratio ranged from 1.5/1 to 8.0/1. With an excess of 5 mM $MgCl_2$, this ratio was shifted to a ratio of 1/6. After $MgCl_2$ addition, no change was observed in the position of the breaking point on the curve. Since the breaking point of the two lines was independent of added $MgCl_2$, the $[Mg^{2+}]/[MgATP]$ and [ATP]/[MgATP] are unrelated to that effect. Furthermore, the breaking point was insensitive to the presence of the nucleotide inhibitors UDP, dGDP and dGTP.

It is possible that the break in the line is the result of an enzyme which uses different kinetic mechanisms at different MgATP concentrations. Glutathione transferase A purified from rat liver gave a similar break in a reciprocal plot when [GSH] was varied and kinetic analysis showed that at low concentrations of GSH, a ping-pong pathway predominated and at high concentrations, an ordered-sequential pathway was observed [13].

No nucleotide was found to significantly activate deoxyguanosine kinase in neonatal mouse skin. Several nucleotides were inhibitory and are listed in order of increasing strength: UDP, dGDP and dGTP. From the estimates of the apparent K_i values for these inhibitors with respect to the MgATP site, and from what is known of intracellular concentrations of nucleotides [14,15] UDP and dGTP would be candidates for physiologically significant inhibitors of deoxyguanosine kinase in neonatal mouse skin.

Of particular interest is the fact that UDP inhibits deoxyguanosine kinase. This compound is a substrate of ribonucleotide reductase and is ultimately converted to dTTP, one of the four substrates for DNA polymerase. Thus, inhibition by UDP may represent an important link in the regulatory relationships between the de novo and salvage pathways for deoxynucleoside triphosphate synthesis.

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