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REASSESSMENT OF THE INTERACTIONS OF GUANYLATE KINASE AND 6-THIOGUANOSINE 5'-PHOSPHATE

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Abstract—Contrary to published studies, 6-thioguanosine 5'-phosphate (6-thioGMP) was found to be a relatively weak inhibitor of the phosphorylation of GMP catalyzed by guanylate kinase. The inhibition constant for 6-thioGMP was 2.3 mM for the enzyme from human erythrocytes. This K_i value is 30-fold larger than previously reported values. The weak inhibition by 6-thioGMP is related to the alternate substrate activity of this compound. It has an apparent K_m value of 2.1 mM and a maximal velocity of 3 per cent of that attainable with GMP as the saturating substrate. The discrepancy between these findings and those previously reported was found to be due to a spectrophotometric artifact that was associated with the high absorbance of 6-thioGMP at the wavelength previously used for the velocity measurements. Guanylate kinase from rat liver, hog brain, Sarcoma 180 tumor cells and Ehrlich ascites tumor cells was also inhibited by 6-thioGMP with a K_i in the mM range.

For a number of years, the inhibition of guanylate kinase* by 6-thioGMP has been proposed to be one of the modes of action of the metabolically activated thiopurines: 6-mercaptopurine, 6-thioguanine and their respective ribonucleosides [1, 2]. Studies with the partially purified enzyme from a number of sources [3–7] have indicated that 6-thioGMP was a competitive inhibitor with respect to GMP with a K_i value in the range of 0.05 to 0.21 mM. Contrary to the results with the isolated enzyme, studies *in vitro* with Sarcoma 180 cells, lymphoma L5178Y cells, H.Ep.2 cells [8–11] or Ehrlich ascites cells [12] have shown that preloading the cells with concentrations of 6-thioGMP up to 0.2 mM has little or no effect upon the incorporation of guanine into GTP.

In an effort to resolve this apparent contradiction, the interactions of 6-thioGMP and guanylate kinase have been re-examined. A spectrophotometric artifact was found to have been present in the earlier enzyme assays. The kinetic constants determined in the absence of this artifact are in accord with the findings of the intact cell studies.

MATERIALS AND METHODS

Materials

Guanylate kinase† was partially purified from human erythrocytes (2.9 U/mg) [5], rat liver (0.15 U/mg) [7], Sarcoma 180 cells (1.1 U/mg) [6] and Ehrlich ascites cells (1.5 U/mg) [13]. Hog brain guanylate kinase (10 U/mg), pyruvate kinase (200 U/mg), lactate dehydrogenase (550 U/mg), and the sodium salts of NADH, ATP and phosphoenolpyruvate were purchased from Boehringer Mannheim Corp. [8-^{14}C]GMP was purchased from Amersham/Searle. 6-ThioGMP was prepared chemically [14] from 6-thioguanosine and enzymatically [15] from 6-thioguanine and was purified as described elsewhere [16]. The two preparations were found to be indistinguishable and to be >99 per cent pure as analyzed by high pressure liquid chromatography.

Enzyme assays

Spectrophotometric assay. Guanylate kinase activity was determined by coupling the formation of the products, ADP and either GDP or 6-thioGDP,‡ to the pyruvate kinase and lactate dehydrogenase mediated oxidation of NADH as described by Agarwal and Parks [7]. Reaction rates were measured in 1-cm path length black-masked quartz semi-micro cuvettes and were monitored at 30° in a thermostated Gilford model 240 spectrophotometer using a full-scale recorder deflection of 0.1 absorbance unit. All measurements were obtained using the tungsten light source with the blue filter§ in the light path. Reaction mixtures contained 100 mM Tris–acetate, pH 7.5; 10 mM $MgCl_2$; 4 mM ATP; 100 mM KCl; nucleoside monophosphate substrate; guanylate kinase; and a coupling system consisting of 0.15 mM NADH,

* *Abbreviations used* are: guanylate kinase = ATP:GMP phosphotransferase, EC 2.7.4.8; 6-thioGMP = 6-thioguansine 5'-phosphate; 6-SeGMP = 6-selenoguanosine 5'-phosphate; U = enzyme unit (1 μmole product formed/min); and M_{ATP} = electrophoretic mobility of ATP.

† Specific activity was determined at 0.1 mM GMP as previously described [6].

‡ Studies indicate that under these conditions the phosphorylation of 6-thioGDP by pyruvate kinase is not rate limiting (unpublished data).

§ Supplied with the spectrophotometer.

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1.5 mM phosphoenolpyruvate, 2.5 U/ml of pyruvate kinase and 3.3 U/ml of lactate dehydrogenase. Other details are described in the figure legends. Reactions were initiated with either guanylate kinase or nucleoside monophosphate after a 3-min preincubation in a 30° water bath. The oxidation of NADH was monitored at 373 nm [$\Delta\epsilon = 3.6\,mM^{-1}cm^{-1}$]. The ϵ_{373} for 6-thioGMP is $0.13\,mM^{-1}cm^{-1}$. All absorbance changes reported in the present study were obtained under conditions of <0.04 per cent stray light. In no case did the absorbance of a reaction mixture exceed 1.2 units at 373 nm.

Radiochemical assay. Reaction mixtures identical to those for the spectrophotometric assay in the absence of the coupling system were used. GMP was replaced by [8-^{14}C]GMP (61 Ci/mole; varied from 0.01 to 0.1 mM). Reactions were initiated with guanylate kinase after preincubation for 3 min at 30° and reactions were terminated after 5 min by the addition of 25 μl of 1.1 M EDTA, pH 9, containing 35 mM GMP and 35 mM GDP carrier. An aliquot (10 μl) of this mixture was spotted on Whatman 3MM paper. The substrate and product were separated by high voltage electrophoresis in 50 mM sodium citrate, pH 3.5, at 3000 volts for 60 min ($M_{ATP} = 100$; $M_{GMP} = 56$; $M_{GDP} = 104$; and $M_{GTP} = 107$). The ultraviolet absorbing spots corresponding to GMP and GDP were cut out and counted for radioactivity as previously described [16].

RESULTS

Spectrophotometric stray light artifacts

In previous studies with guanylate kinase, both the substrate and inhibitory properties of 6-thioGMP were determined spectrophotometrically at 340 nm using a tungsten light source without a filter in the light path [3–7]. Examination of several spectrophotometers revealed that at high absorbance they did not respond in a linear fashion under these conditions (Fig. 1). Three factors which have been reported to be common causes of stray light artifacts appear to have been responsible for this non-linear response.* First was the use of an energy source (tungsten lamp) which has a disproportionate amount of energy with longer wavelength than that used to monitor the reaction rates. Second was the failure to decrease the intensity of this long wavelength energy exiting from the monochrometer by use of the blue filter. Third was the use of a wavelength for monitoring the reaction rates at which the absorbance of the samples was very high. At high absorbance, stray light becomes an appreciable portion of the light energy being measured and thus causes relatively large errors in quantitative measurements.

As previously described by Cavalieri and Sable [18], the error caused by stray light in the determination of absolute absorbances is compounded when measuring absorbance changes. For example, a reaction with an absorbance of 2.1 measured with a spectrophotometer having 0.4 per cent stray light displays a 17 per cent error in absolute absorbance and a 45 per cent error in the observed reaction velocity for a 0.1 absorbance unit decrease. The reaction mixtures previously used in the determination of the inhibition constants for 6-thioGMP had absorbance values at 340 nm ranging from 0.9 for reactions in the absence of 6-thioGMP to 2.1 at the highest 6-thioGMP concentrations. Under these conditions, the values determined for the inhibition constants would be invalid because of the effects of stray light.

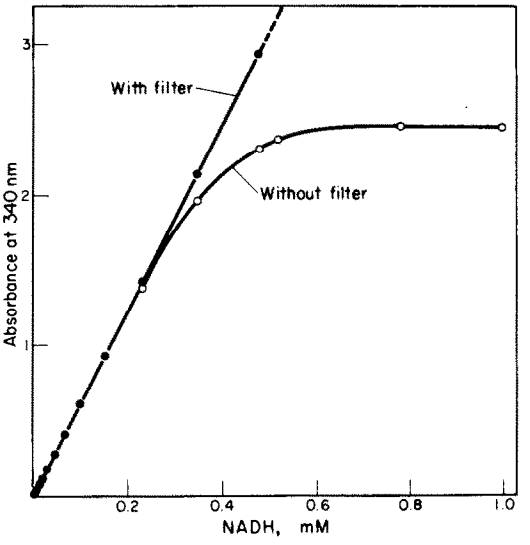

Fig. 1. Effect of stray light on observed absorbance. Absorbance measurements were determined using the tungsten light source with and without the blue filter in the light path. Stray light was 0.4 per cent in the absence of the filter and <0.03 per cent in the presence of the filter.

In the present study, inclusion of the blue filter in the light path decreased the stray light from 0.4 to <0.04 per cent thereby decreasing the error in absolute absorbance measurements to <0.2 per cent. When the reaction rates were monitored at 373 nm

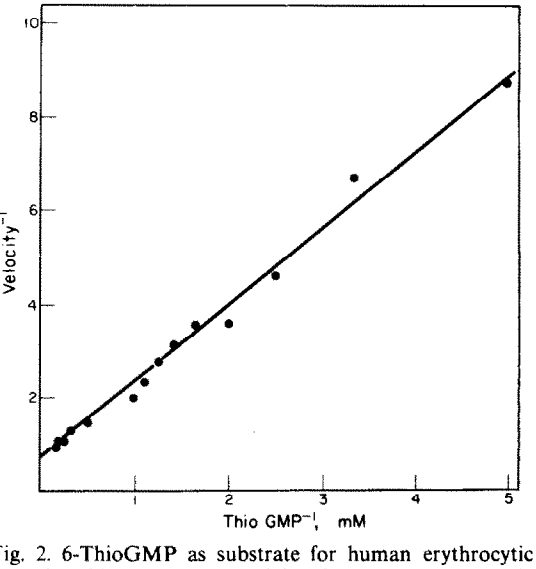

Fig. 2. 6-ThioGMP as substrate for human erythrocytic guanylate kinase as measured by the spectrophotometric assay. Reactions (250 μl) contained 0.04 U guanylate kinase. Initial velocities are expressed as nmoles 6-thioGDP formed/min.

* For a more complete discussion of the causes and effects of stray light, see Ref. 17 and 18.

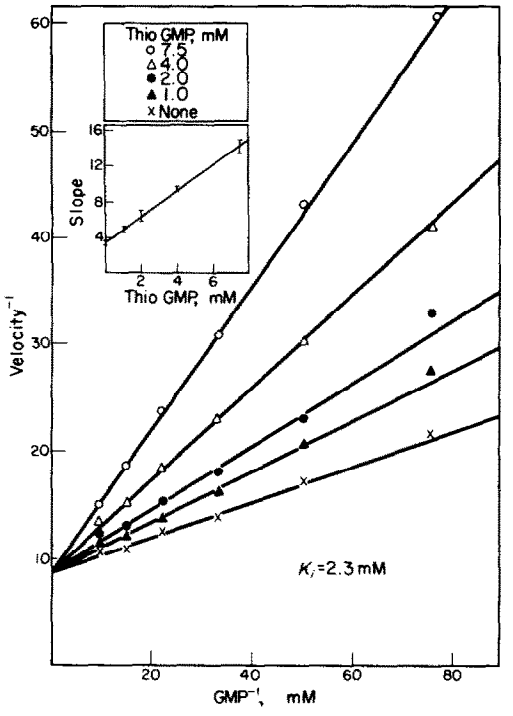

Fig. 3. 6-ThioGMP-mediated inhibition of the conversion of GMP to GDP as measured by the radiochemical assay. Reactions (100 μl) contained 9×10^{-5} U of human erythrocytic guanylate kinase. Initial velocities are expressed as nmoles GDP formed/min. Bars in the secondary plot (see inset) represent the range of slopes obtained from three separate determinations. K_m for GMP = 0.017 mM.

instead of 340 nm, reaction mixtures containing high concentrations of 6-thioGMP had absorbances values of <1.2. Under these conditions, the error in the determination of the reaction rates was decreased from 45 to <0.6 per cent.

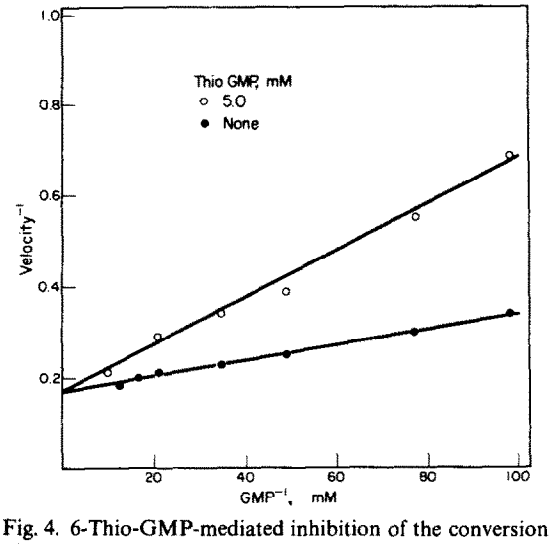

Fig. 4. 6-Thio-GMP-mediated inhibition of the conversion of GMP to GDP as measured by the spectrophotometric assay. Reactions (250 μl) contained 0.005 U of human erythrocytic guanylate kinase. Initial velocities are expressed as nmoles GDP formed/min. K_m for GMP = 0.011 mM.

Substrate activity of 6-thioGMP

In the presence of rate-limiting amounts of erythrocytic guanylate kinase and an excess of pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate, 6-thioGMP was converted to 6-thioGTP. Monitoring the formation of 6-thioGTP and the concomitant oxidation of NADH at 373 nm instead of 340 nm allowed relatively high concentrations of 6-thioGMP (0.2 to 6 mM) to be used without interfering with the accuracy of the assay. 6-ThioGMP was thereby shown to be an alternate substrate for guanylate kinase with a K_m value of 2.1 ± 0.2 mM and a V_{max} of 2.6 ± 0.2 per cent of that observed with GMP (Fig. 2). Preliminary studies with [^{35}S]6-thioGMP support these data. The K_m value for GMP was 0.011–0.017 mM (Figs 3 and 4) which is in agreement with earlier studies [6].

Inhibitory activity of 6-thioGMP

In order to circumvent any possible effects of stray light on the spectrophotometric assay, a radiochemical assay measuring the phosphorylation of [^{14}C]GMP was used. Initial velocities were determined while varying the GMP concentration at several fixed concentrations of 6-thioGMP. An apparent inhibition constant for 6-thioGMP of 2.3 ± 0.6 mM was thereby obtained (Fig. 3). To confirm this finding, the K_i value was determined using the spectrophotometric assay at 373 nm (Fig. 4). The value obtained spectrally, 2.8 ± 0.2 mM, was nearly identical to that obtained radiochemically. The previously reported K_i values for the enzyme from this source [5, 6] were at least an order of magnitude lower than the value determined here.

Preliminary studies with guanylate kinase from hog brain, rat liver, Sarcoma 180 tumor cells and Ehrlich ascites tumor cells also revealed that, contrary to the previously reported values [1, 3–7], the apparent inhibition constants for 6-thioGMP were also in the mM range.

DISCUSSION

The substrate and inhibitory properties of 6-thioGMP with partially purified guanylate kinase which are presented here are quantitatively quite different than those previously reported [3–7].

Previous studies dealing with the stubstrate properties of 6-thioGMP were conducted under conditions of non-linear spectrophotometric response. In addition, the concentrations of 6-thioGMP used were considerably lower than its K_m concentration. Thus, velocities of <1 per cent of GMP instead of 3 per cent were observed [4, 5, 7]. The use of techniques which permit assays to be monitored with higher concentrations of 6-thioGMP have now revealed that the velocity of the phosphorylation of 6-thioGMP by partially purified guanylate kinase approximates that observed in intact erythrocytes incubated with 6-thioguanosine [19].

In studies by Lau and Henderson [12], Crabtree *et al.* [8] and Nelson *et al.* [9–11], preloading of cells with 6-thioGMP was shown to have little or no effect on the conversion of guanine to GTP. Nelson *et al.* [11], in calculations using the concentrations *in vivo* of 6-thioGMP attained and the incorrect inhibition constant of 0.075 mM, have proposed that the lack

of any observed inhibition by 6-thioGMP was due to the fact that only 46 per cent of the guanylate kinase would be inhibited. Thus, this step would not become rate limiting in the conversion of guanine to GTP. This conclusion is further substantiated by recalculation using the inhibition constant of 2.3 mM found in the present study. Actually only 2 per cent of the guanylate kinase would be inhibited under the conditions used. Since the concentration of 6-thioGMP (up to 0.2 mM) found in cells treated with thioguanine or thioguanosine is only about one-tenth of its inhibition constant, instead of 3-fold greater as previously supposed, 6-thioGMP would not be expected to significantly affect guanylate kinase *in vivo*.

In a recent report using the same spectrophotometric assay previously used to determine the inhibition constant for 6-thioGMP, 6-SeGMP has been reported to be a competitive inhibitor of guanylate kinase from rat liver [7], human erythrocytes and S-180 cells [20]. Inhibition constants of approximately 0.1 mM were obtained. In view of the fact that the reaction mixtures used to determine these values had absorbances approaching 2.5 at 340 nm, the validity of these constants is in question. Re-examination of the interaction of 6-SeGMP and guanylate kinase is in progress (laboratory of R. E. Parks, Jr.).

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