INHIBITOR OF PYRIMIDINE METABOLISM FROM TUMOR TISSUES

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SUMMARY

Inhibitors of normal rat liver 5'-nucleotidase and dUMP kinase *in vitro* were found in rapidly proliferating tissues, such as Yoshida sarcoma. Two inhibitors were separated from Yoshida sarcoma by zone electrophoresis, gel filtration on Sephadex G-200 and DEAE-cellulose column chromatography. One inhibited both 5'-nucleotidase and dUMP kinase, while the other inhibited only dUMP kinase. These inhibitors were not detectable in normal rat liver. They were induced in regenerating rat liver and present in rapidly proliferating tissues, such as Yoshida sarcoma and Ehrlich ascites tumor and rat marrow cells. These inhibitors were heat labile. One had a large molecular weight (500,000>) and the other a small molecular weight (*Ca.* 50,000).

INTRODUCTION

The initiation of DNA synthesis is known to be intimately associated with the appearance of elevated levels of enzymes involved in the synthesis. These enzymes include thymidine kinase (1-15), dTMP kinase(1,2,10,14), dTMP synthetase (1,2,5,6), dCMP deaminase (1,2,5,6,14), ribonucleotide reductase (6.16) and DNA polymerase (1,2). The present paper reports that dUMP kinase activity is high in normal rat liver but is low in rapidly proliferating tissues, such as tumor tissues and rat marrow cells. In agreement with the results reported by Kielly (19) on mouse hepatoma and mouse liver, dCMP kinase and CMP kinase activity were lower in hepatoma cells than in normal liver. Thus, it is suggested that the level of this enzyme may be controlled by some regulator (inhibitor and inactivator). Fausto (20), and Fiala and Fiala (21) reported that the elevated enzyme activities of OMP pyrophosphorylase and decarboxylase in regenerating rat liver, and dTMP kinase, dTDP kinase and dCMP deaminase in Ehrlich ascites tumor were inhibited by microsomes of rat liver. Previously it was reported this laboratory that thymidine kinase and

uridine kinase activities in regenerating rat liver were reduced by the microsomal fraction of normal rat liver. It was suggested that the inhibitory effect of this fraction on the kinases may result from the action of phosphatase for the substrate which is less active than in normal rat liver (17). Elford (22) found that the supernatant fraction obtained by ultracentrifugation of an adult rat liver homogenate significantly inhibited the activity of a partially purified preparation of ribonucleotide reductase from Novikoff tumor.

MATERIALS AND METHODS

Preparation of Inhibitor: Yoshida sarcoma cells were grown i.p. in Donryu rats weighing 80-100g for 6 or 7 days. Cells were harvested by centrifugation at the time of active proliferation and washed with cold isotonic saline. Then they were suspended in 4 volumes of ice-cold 0.25 M sucrose containing 10 mM Tris-HCl buffer (pH 7.5), and homogenized with a Teflon homogenizer. The supernatant fraction was obtained by centrifugation at 8,000×g for 30 min and then recentrifugation of the resultant supernatant at 105,000×g for 60 min. The clear supernatant fluid was brought to 30% saturation of ammonium salfate by addition of solid salt and stirred for 60 min. The precipitate formed was removed by centrifugation at 10,000×g for 30 min. More ammonium sulfate was added to the supernatant to 60% saturation. After stirring for 60 min the precipitate formed was collected by centrifugation, dissolved in 10 or 50 mM Tris-HCl buffer (pH 8.0) and dialyzed overnight against the same buffer. This fraction was used as the preparation of inhibitor.

Preparation of dUMP kinase from normal rat liver: Adult male albino rats (Wistar-King strain) weighing 150-200g, were killed by decapitation. The liver was quickly excised and perfused with isotonic saline via the portal vein. Then it was homogenized in 4 volumes of ice-cold 0.25 M sucrose containing 10 mM Tris-HCl buffer (pH 7.5). The supernatant and microsomal fractions were obtained by centrifugation of 20% homogenates for 30 min at 8,000×g and recentrifugation of the resulting supernatant at 105,000×g for

60 min. The final precipitate was used as a preparation of 5'-nucleotidase. The clear supernatant fluid was brought to 60% saturation of ammonium salfate by addition of solid salt and stirred for 60 min. The mixture was centrifuged at 10,000×g for 30 min, and the supernatant was dialyzed overnight against 2 liters of 50 mM Tris-HCl buffer (pH 8.0) with 3 changes of the buffer , and used as a preparation of dUMP kinase.

dUMP Kinase inhibitor activity: Volumes of 50 µl of the preparation of dUMP kinase (20-30 nmoles; specific activity 150 nmoles/mg protein/30 min) and fractions of inhibitor were preincubation at 37° for 20 min, and then mixed with 150 µl of reaction mixture containing 10 µmoles of Tris-HCl buffer (pH 8.0), 4 µmoles of $MgCl_2$, 2 µmoles of ATP and 50 nmoles of [5-^{3}H]dUMP (50,000 cpm) and incubated for 30 min at 37°. The reaction was stopped by immersing the tubes in boiling water for 2 min. The products were separated by chromatography on an ECTEOLA-cellulose column (1×5 cm) as described by Weissman et al.(15) and by us (23). The column was eluted successively with 50 ml portions 0.01 N, 0.05 N and 0.5 N HCl to remove dUMP, dUDP and dUTP, respectively, and radioactivity was determined in an Aloka Liquid Scintillation counter.

5'-Nucleotidase inhibitor activity: The microsomal fraction (0.1 ml, 100-150 nmoles; specific activity 400-500 nmoles) and 0.1 ml of inhibitor were preincubated at 37° for 20 min, and then 50 µl of reaction mixture containing 2 µmoles of substrate (dTMP or dUMP), 2.5 µmoles of $MgCl_2$ and 10 µmoles of Tris-HCl buffer (pH 7.5) was added and mixtures were incubated for 30 min at 37°. The reaction was stopped by adding 1 ml of ice-cold TCA to give a a final concentration of 5%. Inorganic orthophosphate was estimated colorimetrically by the method of Fiske and SubbaRow (18).

Protein assay: Protein concentration was measured by the method of Lowry et al.(24) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

dUMP Kinase and 5'-nucleotidase activities were high in normal rat liver,

but dUMP kinase was very low in rapidly proliferating tissues, such as Yoshida sarcoma, Ehrlich ascites tumor and rat marrow cells. 5'-Nucleotidase activity was also reduced in these tissues, (Table I).

TABLE I

Activities of 5'-nucleotidase and dUMP kinase in various tissues.

5'-Nucleotidase activity was determined in the 8,000×g supernatant fractions and dUMP kinase activity was measured in the 105,000×g supernatant fractions of homogenates of various tissues.

Tissue	5'-Nucleotidase activity *		dUMP Kinase activity **
	dTMP	dUMP	
Normal rat liver	0.630	0.852	102.4
Regenerating rat liver	0.367	0.398	66.3
Rat marrow cells	0.051	—	0.5
Yoshida sarcoma	0.139	0.062	0.9
Ehrlich ascites tumor	0.196	—	0.7

* Pi formed (μmoles/mg protein/30 min)
** dUDP + dUTP formed (nmoles/mg protein/30 min)

TABLE II

Effect of Yoshida sarcoma extract on normal rat liver dUMP kinase.

The 105,000×g supernatant fraction of rat liver (58 μg protein per tube) as dUMP kinase was mixed with various amounts of the 105,000×g supernatant fraction of Yoshida sarcoma.

Yoshida sarcoma extract (μg protein)	dUMP Kinase activity (nmoles)	Per cent inhibition
non	9.05	0
18	4.80	53
36	2.70	79
72	0	100
heated	9.00	0

This suggests that an inhibitors of 5'-nucleotidase and dUMP kinase may be present in these tissues.

So, the effect of the 105,000×g supernatant fraction of Yoshida sarcoma on the phosphorylation of dUMP by the 105,000×g supernatant fraction from normal rat liver was studied. As shown in Table II, normal rat liver dUMP kinase was inhibited by the 105,000×g supernatant fraction of Yoshida sarcoma, when

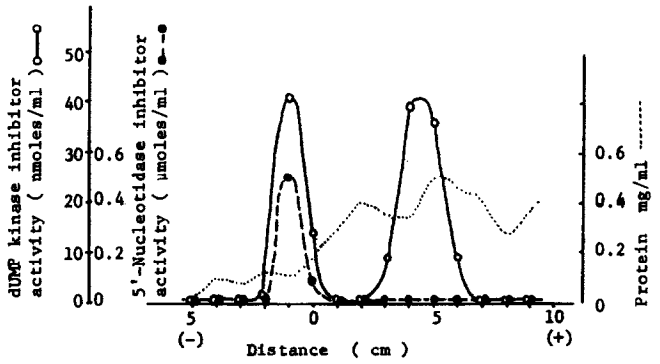

Fig. 1 Zone electrophoretic pattern of the inhibitor of dUMP kinase from Yoshida sarcoma on a Pevikon block.

The Pevikon block measured 30×3×1 cm. Veronal buffer (pH 8.0, ionic strength 0.05) was used throughout. Electrophoresis was carried out at a voltage of 10 V/cm and current of 20 mA. The Pevikon block was cooled by ice during electrophoresis. After electrophoresis the block was cut into 30 segments. Each was extracted with 1 ml of 50 mM Tris-HCl buffer (pH 8.0) and the extracts were used for enzyme and protein estimation.

the later had been heated in boiling water for 3 min., it was no longer inhibitory. The 105,000×g supernatant fraction from Yoshida sarcoma was fractionated with ammonium salfate as described under " Materials and Methods " and subjected to zone electrophoresis on a Pevikon block.

Fig. 1, shows that two peaks of inhibitor activity were separated on zone electrophoresis. One peak, migrating to the cathode, inhibited both 5'-nucleotidase and dUMP kinase, while the other peak, migrating to the anode inhibited only dUMP kinase. These inhibitors from the 105,000×g supernatant fraction were prepared as described in " Materials and Methods ", precipitated with ammonium sulfate (30 to 60% saturation) and applied to a DEAE-cellulose column. The elution profile in Fig. 2, shows two peaks of dUMP kinase inhibitor activity. 5'-Nucleotidase inhibitor activity was eluted only in the unabsorbed fraction.

These fractions of inhibitor had no detectable phosphatase activity on dUMP. The molecular weight was estimated by a modification of the method of Anderson (25). A Sephadex G-200 column was calibrated with proteins of

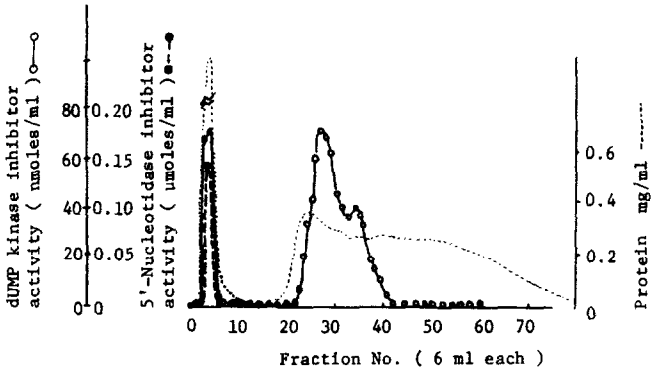

Fig. 2 DEAE-cellulose elution profile of inhibitor from Yoshida sarcoma extracts.

A column of DEAE-cellulose (2×9 cm) was equilibrated with 10 mM Tris-HCl buffer. The column was eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer, in a total volume of 500 ml. The flow rate was 1.5 ml per min and fractions of 6 ml were collected.

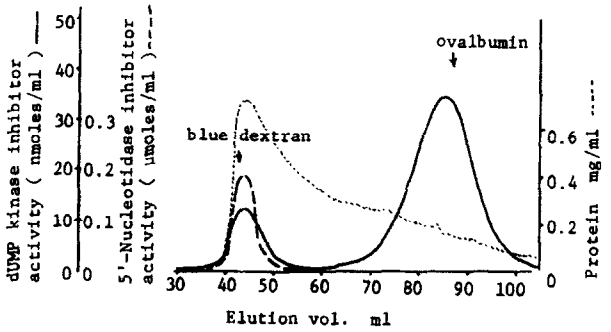

Fig. 3 Gel filtration of the inhibitor of dUMP kinase and 5'-nucleotidase from Yoshida sarcoma on Sephadex G-200.

The Sephadex G-200 column was washed and eluted with 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of approximately 0.5 ml per min, fractions of 3 ml were collected.

known molecular weight, (blue dextran 600,000; and ovalbumin (twice crystallized) 45,000). The fraction of Yoshida sarcoma extract precipitated with ammonium salfate as described in " Materials and Methods " was applied to a column (2 × 50 cm) of Sephadex G-200 equilibrated with 50 mM Tris-HCl buffer (pH 8.0). As seen in Fig. 3, dUMP kinase inhibitor on the Sephadex G-200

column were about 45 ml and 85 ml, respectively, indicating that their molecular weights were about 500,000 > and 50,000. These results indicating that in rapidly proliferating tissues, 5'-nucleotidase and dUMP kinase activities are regulated by an inhibitor. This inhibitor was not detectable in normal rat liver, but it was found in regenerating rat liver.

These inhibitors may be induced in rapidly proliferating tissues, and it seems likely that as inhibitors of 5'-nucleotidases and dUMP kinase they are important in regulating DNA synthesis in rapidly proliferating tissues.

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