THE PRESENCE OF AN INHIBITOR OF tRNA SULFURTRANSFERASE IN MORRIS HEPATOMAS*

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SUMMARY: A tRNA sulfurtransferase has been isolated from the 160,000g supernatant of Buffalo rat liver and a number of Morris hepatomas. This enzyme catalyzes the transfer of labeled sulfur from [^{35}S]β-mercaptopyruvate to tRNA in the presence of ATP and magnesium ion. All 6 Morris hepatomas examined (9618A$_2$, 7777, 5123TC, 7800, 5123B, and 7787) have a lower specific activity of tRNA sulfurtransferase than their host liver or normal liver. The decrease in enzyme activity is roughly proportional to the growth rates of the tumors. The faster the growth rate, the greater the decrease in tRNA sulfurtransferase activity. The lower enzyme activity is due in part to the presence of an inhibitor in the tumor supernatants. Studies with the Morris hepatoma 5123TC indicates that the inhibitor is dialyzable and heat-stable, with a molecular weight below 5000.

Thionucleotides are among the minor components of bacterial, yeast, and mammalian transfer RNA (1-5). Their presence in or near the anticodon of certain tRNAs have been shown to be essential to specificity of codon recognition and efficient binding to ribosome-mRNA complexes (5-7). Recently, we reported the isolation of an enzyme system from the soluble cytoplasm of rat brain which is capable of transferring the labeled sulfur from [^{35}S]β-mercaptopyruvate to tRNA, producing several thionucleotides (8). This was the first report of tRNA sulfurtransferase isolated from mammalian tissue, though it had been described in bacteria (9-11). We now report the isolation of a similar tRNA sulfurtransferase from the cytosol of Buffalo rat liver and a number of transplantable Morris hepatomas. The specific activity of this enzyme is uniformly decreased in the hepatomas compared to their host liver or normal control liver. Evidence is presented that this decrease in tRNA sulfurtransferase activity is due in part to the presence of an inhibitor in the tumor cytosol.

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MATERIALS AND METHODS

Animals and Tumors. All animals used in this study were adult male Buffalo rats purchased from Simonsen Laboratories, Gilroy, Calif. The Morris hepatomas were originally induced by chemical carcinogens and were maintained by serial bilateral transplantations in the thigh muscles of male Buffalo rats (12). The tumors studied fell into 3 groups: 1) the fast-growing hepatomas 9618A$_2$ and 7777; 2) the intermediate-growing hepatomas 5123TC, 7800, and 5123B; 3) the slow-growing hepatoma 7787. The tumor-bearing animals were sacrificed when their hepatoma transplants grew to a usable size (about 2 to 3 cm) and necrosis was still minimal. Normal adult male Buffalo rats were on occasions sacrificed along with the tumor-bearing animals to provide normal control liver.

Preparation of Enzymes. The rats were killed by exsanguination through the abdominal aorta while under light ether anesthesia. The hepatoma transplants and the host (or normal) liver were removed rapidly. All tumors were sliced and immediately dissected free of nontumorous and necrotic tissue. Thereafter all procedures were carried out at 4°C. The tumor or liver from each animal was homogenized in chilled 0.14 M KCl - 0.02 M Tris (pH 7.4) in the amount of 10 ml/g of tissue, using a glass homogenizer and a mechanically driven Teflon pestle. Afterward the homogenate was centrifuged twice at 1,600g for 10 min, once at 33,000g for 20 min, and again at 160,000g for 60 min. The resulting 160,000g supernatant was stored at -75°C until assay.

Assay of tRNA Sulfurtransferase Activity. The *in vitro* transsulfuration reaction uses the 160,000g supernatant of rat liver or hepatoma as enzyme, [^{35}S]β-mercaptopyruvate as sulfur donor, and tRNA as sulfur acceptor. The standard assay mixture is 0.5 ml in volume and contains 5 nmoles of ammonium [^{35}S]β-mercaptopyruvate (specific activity 350 - 400 mCi/mmole), 0.5 mg of yeast tRNA, 0.02 ml (unless specified otherwise) of rat liver or hepatoma 160,000g supernatant, 50 μmoles of Tris (pH 7.4), 1 μmole of ATP, 3 μmoles of $MgCl_2$, and 1 μmole of β-mercaptoethanol. The standard control consists of the above mixture with 25 μmoles of EDTA added. This control is based on the fact that the reaction has an absolute requirement for magnesium ion; in the presence of EDTA, which binds magnesium ion, the reaction cannot take place. The reaction mixture is incubated at 37°C for 20 min. Thereafter the tRNA is recovered for determination of its ^{35}S-labeling by scintillation counting as described previously for the rat brain tRNA sulfurtransferase (8).

RESULTS AND DISCUSSION

The requirements of the transsulfuration reaction are illustrated with normal liver 160,000g supernatant in Table 1. Radioactive label is incorporated into tRNA when [^{35}S]β-mercaptopyruvate is incubated in a complete reaction mixture with the enzyme. Incorporation is reduced to baseline levels when enzyme is omitted, when heated enzyme is used, or when Pronase is added to the reaction mixture. ^{35}S transfer to tRNA also requires the presence of ATP and magnesium ion. When ATP or magnesium ion is omitted, or when the magnesium ion is bound by the addition of EDTA, no ^{35}S transfer occurs. Formation of labeled product requires tRNA, and does not take place in its absence. Adding RNase abolishes transsulfuration, but adding DNase has no such effect, indicating that the ^{35}S is incorporated into tRNA.

The tRNA sulfurtransferase activities of a variety of Morris hepatomas and their corresponding host livers are compared in Table 2. All tumors show a decrease in enzyme

TABLE 1. Requirements of Transsulfuration Reaction with Liver 160,000g Supernatant as Enzyme, [^{35}S]β-Mercaptopyruvate as Sulfur Donor, and Yeast tRNA as Sulfur Acceptor

Reaction Mixture	^{35}S Incorporated into tRNA	
	CPM	pmoles
Complete	4393	5.40
No enzyme	541	0.67
Heated enzyme	529	0.65
Add Pronase	579	0.71
No ATP	675	0.83
No $MgCl_2$	588	0.72
Add EDTA	627	0.77
No tRNA	579	0.71
Add RNase	805	0.99
Add DNase	4308	5.29

The complete reaction mixture and assay procedure were as described under Materials and Methods. Where indicated, one of the following was also present: 1) 0.02 ml of heated (5 min at 100°C) liver 160,000g supernatant in place of the same amount of unheated enzyme, 2) 25 μg of Pronase, 3) 25 μmoles of EDTA, 4) 50 μg of pancreatic RNase and 10 μg of T_1 RNase, and 5) 50 μg of pancreatic DNase.

activity compared to the host livers. On the whole, the decrease is proportional to the growth rates of the tumors. The hepatomas with the fastest growth rates (9618A_2 and 7777), and therefore the shortest transfer times, show the greatest decrease in tRNA sulfurtransferase activity. Those tumors of intermediate growth rates (5123TC, 7800, and 5123B) have less striking decrease in activity, while the slowest-growing hepatoma (7787) shows the least decrease in activity compared to the host liver.

Studies with Morris hepatomas of fast growth rates (exemplified by hepatoma 9618A_2 in Table 3) and of intermediate growth rates (exemplified by hepatoma 5123B in Table 3) indicate that the decrease in tRNA sulfurtransferase activity is due to an enzyme inhibitor in the tumor supernatants, since in each instance the enzyme activity in the host liver supernatant can be suppressed by the addition of tumor supernatant. This suppression can in part be overcome by the addition of more host liver supernatant (Table 3).

TABLE 2. Comparative Study of tRNA Sulfurtransferase Activities in Liver and Morris Hepatomas

Hepatoma	Transfer Time (Months)	Specific Activity in pmoles of ^{35}S Incorporated into tRNA per mg Protein		
		Host Liver	Tumor	Tumor/Host Liver (%)
Fast growth rate				
9618A$_2$	0.6	24.91	1.54	6.2
7777	0.9	24.75	1.99	8.0
Intermediate growth rate				
5123TC	1.2	22.49	3.78	16.8
7800	1.3	24.56	4.32	17.6
5123B	1.6	24.56	5.33	21.7
Slow growth rate				
7787	6.3	24.80	11.19	45.1

The reaction mixture and the assay procedure were as given under Materials and Methods. The 160,000g supernatants of each hepatoma and its host liver were compared with respect to tRNA sulfurtransferase activity. Values for specific activity were calculated from ^{35}S incorporation data obtained with the standard assays and protein concentrations determined by the method of Lowry *et al.* (13).

In contrast, in the case of the slow-growing hepatoma 7787, when equal volumes of liver and tumor enzymes are combined, the ^{35}S incorporation into tRNA is nearly additive (Table 3), suggesting that in this tumor, either no inhibitor is present, or the inhibitor is present at an insignificant level compared to that in other tumors.

The search for tRNA sulfurtransferase inhibitor in the various hepatomas is summarized in Table 4. Inhibitor is found in the hepatomas of fast and intermediate growth rates, but not in the single slow-growing hepatoma we have examined.

To determine whether the inhibitor could be separated from the hepatoma enzymes, portions of each tumor enzyme were dialyzed (Table 5). The specific activities of all tumor enzymes rise after dialysis for 4 hr. But with the exception of the hepatoma 5123B, the dialyzed tumor enzymes still have activities below that of the normal liver enzyme. Due to the relative instability of tRNA sulfurtransferase at 4°C, the tumor enzymes cannot

TABLE 3. Assay for Inhibitor in Three Morris Hepatomas of Different Growth Rates

Hepatoma	Transfer Time (Months)	Volume of Enzyme (ml)		^{35}S Incorporated into tRNA	
		Host Liver	Tumor	Net CPM	pmoles
$9618A_2$	0.6	0.01	——	1836	2.34
		0.02	——	4371	5.57
		0.03	——	5007	6.38
		——	0.01	158	0.20
		0.01	0.01	202	0.26
		0.02	0.01	666	0.84
		0.03	0.01	1062	1.35
5123B	1.6	0.01	——	2128	2.71
		0.02	——	4617	5.88
		0.03	——	5591	7.12
		——	0.01	565	0.72
		0.01	0.01	707	0.90
		0.02	0.01	1429	1.82
		0.03	0.01	1987	2.53
7787	6.3	0.02	——	4051	5.16
		——	0.02	1868	2.38
		0.02	0.02	5566	7.09

The reaction mixture and assay procedure were described under Materials and Methods. Volume of enzyme given above refers to the amount of 160,000g supernatant used in each instance.

be dialyzed exhaustively without loss of activity.

Table 6 shows that the inhibitor is also heat-stable. While the tRNA sulfurtransferase activity in the tumor supernatants can be abolished by heating, the inhibitory activity is essentially unaffected.

We next attempted to isolate the inhibitor from the tumor supernatant by column

TABLE 4. Presence of tRNA Sulfurtransferase Inhibitor in Various Morris Hepatomas

Hepatoma	Transfer Time (Months)	tRNA Sulfurtransferase Inhibitor	
		Demonstrable	Not Demonstrable
$9618A_2$	0.6	+	
7777	0.9	+	
5123TC	1.2	+	
7800	1.3	+	
5123B	1.6	+	
7787	6.3		+

TABLE 5. Dialyzability of tRNA Sulfurtransferase Inhibitor

Enzyme		Specific Activity in pmoles of ^{35}S Incorporated per mg Protein	% of Normal Liver
Normal Liver		22.49	100.0
$9618A_2$	undialyzed	1.53	6.8
	dialyzed	2.66	11.8
7777	undialyzed	1.57	7.0
	dialyzed	3.35	14.9
5123TC	undialyzed	3.46	15.4
	dialyzed	11.60	51.6
7800	undialyzed	4.24	18.9
	dialyzed	7.31	32.5
5123B	undialyzed	5.11	22.5
	dialyzed	22.79	101.3

The assay mixture and procedure were given under Materials and Methods. All enzymes refer to 160,000g supernatants. Dialysis of the tumor enzymes was carried out against 2000 volumes of 0.02 M Tris (pH 7.4) at 4°C for 4 hr. Normal liver enzyme and undialyzed tumor enzymes were left standing at 4°C for the same length of time.

TABLE 6. Heat-Stability of tRNA Sulfurtransferase Inhibitor

Enzyme	^{35}S Incorporated into tRNA	
	Net CPM	pmoles
Normal liver	3672	4.52
Undialyzed 9618A_2	152	0.19
Heated 9618A_2	8	0.01
Normal liver + Undialyzed 9618A_2	553	0.68
Normal liver + Heated 9618A_2	597	0.74
Undialyzed 7777	211	0.26
Heated 7777	0	0.00
Normal liver + Undialyzed 7777	496	0.61
Normal liver + Heated 7777	500	0.62
Undialyzed 5123TC	541	0.67
Heated 5123TC	-28	——
Normal liver + Undialyzed 5123TC	716	0.88
Normal liver + Heated 5123TC	360	0.44
Undialyzed 7800	594	0.73
Heated 7800	0	0.00
Normal liver + Undialyzed 7800	983	1.21
Normal liver + Heated 7800	807	0.99
Undialyzed 5123B	661	0.84
Heated 5123B	-76	——
Normal liver + Undialyzed 5123B	917	1.13
Normal liver + Heated 5123B	948	1.17

The assay mixture and procedure were as described under Materials and Methods. All enzymes refer to 160,000g supernatants. Where indicated, the tumor supernatant was heated at 100°C for 5 min and the coagulated protein was removed by centrifugation prior to use. The volume of each enzyme or heated tumor supernatant used was 0.02 ml.

chromatography, using the hepatoma 5123TC as a source of the inhibitor. The 160,000g tumor supernatant was first heated and then centrifuged to remove the coagulated protein. The deproteinated supernatant was applied to a 108 x 1 cm column of Sephadex G-25 and eluted with 0.05 M NH_4HCO_3 (pH 8.6) - 0.0014 M β-mercaptoethanol. One-ml fractions were collected and their A_{280} and A_{260} were determined. Fractions were pooled, lyophilized, resuspended in water, and assayed for their inhibitory effect on

normal liver enzyme. The results are summarized in Figure 1. The inhibitor from this tumor elutes consistently between fractions 86 and 95. Fractions 86-90 cause nearly 70% inhibition and fractions 91-95, nearly 80% inhibition of the tRNA sulfurtransferase activity of the normal liver supernatant. Other fractions have little or no effect on the liver enzyme. Since the inhibitor elutes beyond the void volume on Sephadex G-25 chromatography, its molecular weight is below 5000.

In conclusion, all 6 Morris hepatomas examined have a lower specific activity of tRNA sulfurtransferase than their host livers. For the slow-growing hepatoma 7787, no inhibitor is found in the cytosol, suggesting that in this instance, the decrease in activity is due solely to a lower enzyme level in the tumor. For those hepatomas of fast

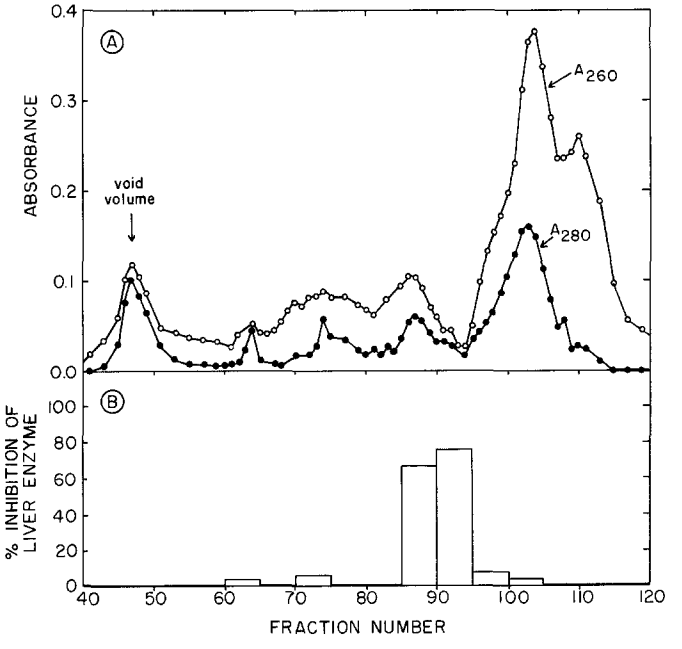

Figure 1. Sephadex G-25 chromatography of tRNA sulfurtransferase inhibitor derived from hepatoma 5123TC (A) and assay of its inhibitory effect on normal liver enzyme (B). Three ml of 160,000g supernatant from hepatoma 5123TC was first heated at 100°C for 5 min and then centrifuged to remove the coagulated protein. The deproteinated supernatant was applied to a 108 x 1 cm column of Sephadex G-25 and eluted with 0.05 M NH_4HCO_3 (pH 8.6) - 0.0014 M β-mercaptoethanol. One-ml fractions were collected and their A_{280} and A_{260} were determined. Every 5 fractions were pooled, lyophilized, resuspended in 1 ml of water, and assayed for their inhibitory effect on normal liver enzyme, using the standard reaction mixture described under Materials and Methods. The volume of normal liver enzyme or resuspended pooled fractions added to the reaction mixture was 0.02 ml.

and intermediate growth rates, the decrease in tRNA sulfurtransferase activity is due in part to an inhibitor present in the supernatants. The inhibitor is a small, dialyzable, and heat-stable molecule. Because attempts to remove the inhibitor by exhaustive dialysis cause a loss of tRNA sulfurtransferase activity, it is uncertain to what extent the decrease in transsulfuration is due to the inhibitor as opposed to a lower level of the enzyme.

Whether this inhibitor of the in vitro transsulfuration reaction has a significance in vivo is not known. One way of answering this question is to analyze the tRNAs from the rat liver and the hepatomas to determine whether there are any quantitative or qualitative differences in their contents of thionucleotides. The function of thionucleotides in tRNA is gradually being elucidated. The 2-thiouridine derivatives found in the first position of the anticodon of certain yeast, E. coli, and rat liver tRNAs have been shown to play a role in the specificity of codon recognition through prevention of wobbling (5,6). Similarly, a thiolated adenosine derivative found adjacent to the 3'-end of the anticodon of an E. coli tRNA has been shown to control the efficiency of binding to ribosome-mRNA complexes (7). The presence of an inhibitor in the hepatomas which suppresses tRNA thiolation could therefore have serious consequences on tRNA function.

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