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GUANINE AMINOHYDROLASE IN RAT AND MOUSE RED CELLS: A POTENT INHIBITOR OF GUANYLATION OF tRNA

WALTER R. FARKAS* and RAM D. SINGH**

University of Tennessee, Memorial Research Center, Knoxville, Tenn. 37920 (U.S.A.)

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Summary

1. The red blood cells of mice and rats contained guanine aminohydrolase (EC 3.5.4.3). This enzyme was not present in rabbit, sheep or human red blood cells.

2. The enzyme from rat blood cells was separated into two activities by column chromatography on DEAE-cellulose. Both isozymes were labile but it was possible to show that the more abundant enzyme followed Michaelis—Menten kinetics, had an apparent K_m of $4.0 \cdot 10^{-6}$ M and was not activated by GTP nor inhibited by allantoin.

3. We believe, therefore, that guanine aminohydrolase was the protein in rat and mouse red blood cells that inhibited the enzyme (in rabbit reticulocytes) responsible for guanylation of tRNA.

Introduction

Recently a new and novel type of modification of tRNA has been reported in rabbit reticulocytes [1,2]. The modification, which has been termed "guanylation", is novel in that it involves the replacement of a base within the polynucleotide chain of tRNA by guanine. The reaction is highly specific for the tRNA molecule modified (in rabbit reticulocytes guanylation occurs in $tRNA_{II}^{(His)}$ and in no other tRNA) [1—3] and also for the position within the polynucleotide chain undergoing modification (after T_1 or pancreatic RNAase digestion of tRNA guanylated with labeled guanine only a single oligonucleotide was labeled) [2].

An early study showed that reticulocytes of rabbits, sheep and man but not those of rats or mice carry out the guanylation reaction [2]. The guanylat-

Abbreviation: TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

* To whom correspondence should be sent.

** Present address: University of Cincinnati Medical Center, Cincinnati, Ohio.

ing enzyme has recently been partially purified and it has further been shown that mouse and rat hemolysates contained an inhibitor of tRNA guanylation [3]. Since tRNA and the enzymes that modify it have been implicated in such important phenomena as viral infection [4], cell development [5], hormone action [6] and tumorogenesis [7], the present study was undertaken to characterize the tRNA guanylation inhibitor present in rodent cells. The results showed that the inhibition was not due to a factor specific for the guanylation reaction but was due to a high level of the enzyme guanase (guanine aminohydrolase, EC 3.5.4.3) in rat and mouse reticulocytes. There were in fact two isozymes for guanase in rat reticulocytes, neither of which were activated or inhibited by GTP.

Materials and Methods

[8-^{14}C]Guanine 40 Ci/mole and [8-^{14}C]guanosine 50 Ci/mole were purchased from Schwarz/Mann Corporation and New England Nuclear Corporation respectively. Omnifluor was obtained from New England Nuclear. Rabbit liver guanase was purchased from Sigma Chemical and had a specific activity of 0.10 units/mg protein. Milk xanthine oxidase had a specific activity of 14 units/mg and was obtained from Worthington Biochemicals.

Preparation of hemolysates

When reticulocytes were used they were obtained by the phenylhydrazine technique [2,3]. Human reticulocytes were obtained from patients with elevated reticulocyte counts. One patient had hemoglobin SC disease and a second was admitted for acute alcoholism. Cells were obtained by cardiac puncture from rabbits, mice and rats and venous puncture from sheep. The cells were sedimented at 750 × *g* to remove the plasma, washed twice with cold isotonic saline and shock-lysed by the addition of an equal volume of cold glass-distilled water. Unlysed cells and stroma were removed by centrifugation at 12 000 × *g*. The stroma from sheep reticulocytes did not pack tightly after centrifugation and only the upper one-third of the centrifuged hemolysate was used.

Preparation of the tRNA-guanylating enzyme

A lysate of rabbit reticulocytes was sedimented at 105 000 × *g* after stroma had been removed. The tRNA was then removed from this post-ribosomal supernatant as follows [8]: a suspension of Whatman DE-32 was centrifuged in a clinical centrifuge tube to a packed resin volume of 3.5 ml. The 105 000 × *g* supernatant was dialyzed against 0.30 M KCl. 6 ml of the dialyzed hemolysate were added to the DE-32 and the mixture stirred in an ice bath for 5 min. The resin was removed by centrifugation. The tRNA-free lysate was dialyzed against H_2O and used as the source of tRNA-guanylating enzyme.

Assay for the inhibitor of the tRNA-guanylating enzyme

The reaction mixture contained 0.20 ml of guanylating enzyme (16.4 mg of protein), 10 μmoles of TES, pH 7.4; 55 μmoles of KCl, 1.9 A_{260} units of yeast tRNA and 0.035 μmoles of [^{14}C]guanine and mouse reticulocyte lysate containing about 3.0 mg of protein in a volume of 0.50 ml. The solution was

incubated at 37°C for 3 h. At the end of the incubation period the tubes were chilled to 0°C and 1 ml of 0.1 M sodium acetate, pH 5.0—0.1 M NaCl—0.01 M EDTA was added followed by 2 volumes of water-saturated phenol [9]. After shaking for 5 min the phases were separated and the aqueous phase collected by aspiration. The phenol phase was washed with an equal volume of the acetate buffer and the aqueous phases were pooled. The tRNA was precipitated with 2 volumes of 95% ethanol. After storage at −15°C for at least 2 h the tRNA was collected by centrifugation and dried in vacuo. The powder was dissolved in 0.40 M ammonium acetate, pH 8.2, and adsorbed to a DEAE-cellulose column [1]. The column was washed with the 0.40 M ammonium acetate buffer until all radioactive material except the guanylated tRNA was eluted. The tRNA was then eluted with 1.0 M NaCl in 0.40 M ammonium acetate, pH 8.2. The absorbance of the tRNA at 260 nm was determined and then the tRNA was precipitated by the addition of trichloroacetic acid to 7.5%. The precipitated tRNA was collected on glass fiber filters and the radioactivity determined with a liquid scintillation counter [2]. Incorporation was expressed as count/min/A_{260} unit.

Spectrophotometric assay for guanase in reticulocyte lysates

The assay for guanase was a modification of the method described by Kalckar [10] and is based on the spectrophotometric determination of uric acid produced when guanine is incubated with guanase in the presence of excess xanthine oxidase [10,13]. The reaction mixture contained 0.03 μmoles guanine (added from a stock solution that was 1.0 mM with guanine dissolved in 50 mM HCl), 6.0 μmoles Tris—HCl, pH 8.0, 0.025 units of xanthine oxidase and the appropriate amount of lysate in a volume of 1.0 ml. The reaction was monitored at 290 nm with a Beckman Acta II spectrophotometer.

Chromatography of red cell guanase on DEAE-cellulose

The ribosomes and tRNA were removed from a hemolysate of rat reticulocytes and 0.10 ml of the hemolysate was diluted with 0.90 ml of 25 mM sodium phosphate, pH 7.0. The diluted hemolysate was adsorbed to a DEAE cellulose column (1 cm × 34 cm) that had been equilibrated with 50 mM sodium phosphate (pH 7.0). The column was eluted with a linear gradient from 50 mM sodium phosphate (pH 7.0) to 400 mM sodium phosphate (pH 7.0) at a flow rate of 1 ml/min at 4°C. At the start of the elution both buffer reservoirs contained 100 ml of the respective buffers. Fractions of 3.6 ml were collected and monitored for absorbance at 280 nm and for guanase activity.

Analysis

Guanine, xanthine and uric acid were separated by descending paper chromatography with a solvent of *n*-butanol, 0.1 M HCl, ethyleneglycol monoethyl ether, 4 : 1 : 1. When radioactive compounds were chromatographed, the paper was cut into 1 cm strips and the radioactivity determined by placing the strips into vials containing 10 ml of omnifluor dissolved in toluene (4 g/l). Protein (hemoglobin) concentration of hemolysates was determined with Drabkin's reagent [11]; otherwise, protein was determined by the method of Warburg and Christian [12].

Results

Species specificity for the guanylation of tRNA and demonstration of an inhibitor in mouse and rat lysates

Lysates prepared from rabbit, sheep and human reticulocytes were able to carry out the guanylation of tRNA but the preparations from mouse or rat were unable to carry out this reaction. This observation is identical to the results observed with intact reticulocytes of these species [2]. When rodent reticulocyte extracts were added to rabbit reticulocytes there was a marked inhibition of the guanylation reaction. Guanosine as well as guanine served as a substrate for the tRNA-guanylating enzyme and the uptake of the nucleoside was inhibited by the rodent lysates as well. Guanosine was not incorporated into tRNA by the rodent lysate.

Characterization of the inhibitor as a protein

The inhibitor was found to have the following properties: it was non-dialyzable, heat labile, destroyed by phenol extraction and refractory to pancreatic RNAase. The insensitivity to RNAase was demonstrated by incubating the inhibitor with matrix bound RNAase [3] followed by removal of the insoluble RNAase by filtration prior to assaying the inhibitor in the tRNA-guanylating enzyme assay using rabbit hemolysate. These observations indicated that the inhibitor was probably a protein.

Identification of the inhibitor as the enzyme guanase

The requirements for the tRNA-guanylation reaction are simple. The only requirement of the enzyme are guanine, a tRNA that is capable of guanine acceptance and a monovalent cation [3]. A likely explanation of the inhibition is the destruction of one of the substrates of the guanylating enzyme rather than the specific inhibition of the enzyme. We decided to test this possibility before embarking on the purification of the inhibitor. The substrates tRNA and [^{14}C]guanine were incubated separately with the mouse lysate. We then removed the protein, recovered the tRNA and guanine, respectively, and tested them as substrates for the rabbit reticulocyte-guanylating enzyme. The data in Table I clearly show that whereas the recovered tRNA was an active guanine acceptor, the guanine completely lost its ability to be a substrate in the tRNA-guanylating reaction. Table I also shows that [^{14}C]guanine did not disappear when incubated with rabbit rather than mouse reticulocytes.

Since guanine was converted to a compound that could not be utilized by the tRNA-guanylating enzyme it became compelling to identify the product(s) of this reaction. [^{14}C]Guanine was incubated with a mouse hemolysate. At the end of the incubation, the protein was removed by acid precipitation and the supernatant analyzed by paper chromatography in a solvent that readily separated guanine, xanthine and uric acid. Incubation of guanine in mouse hemolysate resulted in almost complete conversion of guanine to xanthine and, to a much lesser extent, uric acid, whereas incubation of [^{14}C]guanine with a rabbit hemolysate did not result in disappearance of guanine. A similar experiment with rat hemolysates also brought about conversion of guanine to xanthine but incubation with sheep or human hemolysates resulted in recovery of

TABLE I

EFFECT OF MOUSE LYSATE ON THE SUBSTRATES OF THE GUANYLATION REACTION

96 A_{260} units of yeast tRNA were incubated at 37°C for 1 h with 1.0 ml mouse lysate in 15 mM TES (pH 7.4), final volume is 22 ml. The tRNA was recovered by phenol extraction and ethanol precipitation [1]. It was then dialyzed against H_2O and assayed with the rabbit reticulocyte tRNA-guanylating enzyme. 13 μCi of [^{14}C]guanine was similarly incubated and the protein precipitated by the addition of HCl to a concentration of 2 M. After removal of the protein precipitate the solution was lyophilized and the guanine dissolved in 0.01 M HCl prior to assay.

Incubation	[^{14}C] Guanine incorporation (cpm/A_{260})
tRNA + rabbit lysate	437
tRNA + mouse lysate	449
[^{14}C] Guanine + rabbit lysate	433
[^{14}C] Guanine + mouse lysate	5

only [^{14}C]guanine from the incubation medium. These results suggested that the strong inhibition of tRNA-guanylating enzyme by rodent lysates was due to a potent guanase activity which resided in rodent red blood cells but was not present in the reticulocytes of the other species investigated. The identification of the tRNA-guanylation inhibitor as the enzyme guanase was confirmed by two additional experiments. First, guanase activity was assayed in hemolysates prepared from reticulocytes of different organisms. Only those species (rat and mouse) which were inhibitors also showed significant guanase activity. Finally, mouse hemolysates were heated to different temperatures and assayed in parallel for tRNA-guanylation inhibition and for guanase activity. The results showed that the two activities have almost identical susceptibilities to heat denaturation.

Properties of rat reticulocyte guanase

Fig. 1 shows that two guanase isozymes were resolved when rat reticulocyte lysates were chromatographed on DEAE-cellulose. The peaks were designated guanases A and B, respectively. Both of these enzymes were very labile after purification on DEAE-cellulose. The enzymes were unstable at −40°C, −15°C, 4°C and 25°C and could not be stabilized by 10 mM mercaptoethanol or bovine serum albumin. Guanase A followed Michaelis–Menten kinetics (Fig.

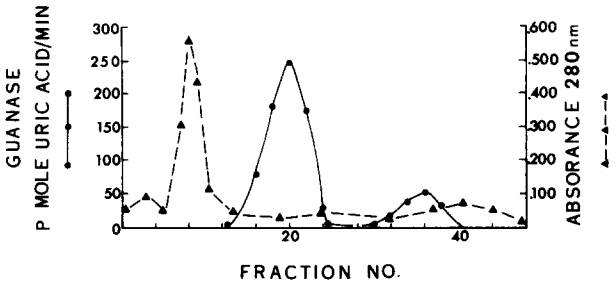

Fig. 1. Separation of two guanase activities from rat reticulocytes on DEAE-cellulose. Absorbance at 280 nm (▲- - - - -▲); guanase activity in pmoles uric acid formed per min (●———●).

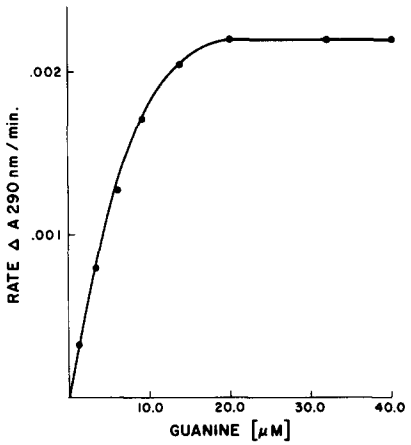

Fig. 2. Effect of varying guanine concentration on the rate of the reaction catalyzed by guanase A.

2), and an apparent K_m of $4.0 \cdot 10^{-6}$ M was calculated for guanine from a Lineweaver—Burk plot (not shown). The greater instability of guanase B precluded a similar study with that isozyme. We were able, however, to do a study of the effect of GTP and the end product of purine metabolism of these cells, allantoin, on the catalytic rates of both isozymes. Neither isozyme was affected by 24 μM GTP or 1.2 mM Mg^{2+}. Guanase A was not affected by 0.24 mM allantoin.

Discussion

The inhibition of guanylation of tRNA by rat and mouse lysates is clearly due to a potent guanase activity in these cells rather than to a specific inhibitor. The question of whether rat and mouse reticulocytes carry out guanylation has not yet been answered. The enzyme, guanase, is highly specific for its substrate and will not react with guanosine or GMP [15]. Therefore, we explored the possibility that some guanine derivative rather than guanine was the substrate for the tRNA guanylating enzyme in rodent reticulocytes. Guanosine, 3′-GMP, 5′-GMP, GDP and GTP were not incorporated into tRNA by mouse reticulocyte hemolysates. This finding is consistent with our previous report that when rabbit reticulocytes are incubated with uniformly labeled guanosine only the purine ring and not the ribose moiety is incorporated into tRNA [3]. Guanosine must be cleaved to guanine and ribose prior to the incorporation of the guanine moiety into reticulocyte tRNA. Modification of tRNA occurs during cell differentiation [5,14] and it is possible that guanylation in the rodent reticulocytes occurs at a time in cell development prior to the synthesis of guanase. The finding that guanase is the inhibitor of the tRNA-guanylating enzyme is somewhat analogous to the discovery that an apparent inhibitor of tRNA methylase in rabbit and rat liver, kidney and pancreas is in fact the enzyme glycine *N*-methyltransferase [16]. Both enzymes remove a low molecular weight substrate of the appropriate tRNA modification enzyme, *S*-adenosylmethionine or guanine. The analogy is not perfect, however, since *S*-adenosylhomocysteine, the product of glycine *N*-methyltransferase is also a potent in-

hibitor of tRNA methylase. Xanthine, the major product of the guanase reaction, is neither an inhibitor or a substrate of the tRNA-guanylating enzyme [2]. Glycine *N*-methyltransferase is found in adult but not in fetal liver and it is possible that guanase may not occur in immature erythroid precursor cells but is found in circulating reticulocytes.

The species and cellular distribution of guanase is complicated. This enzyme is found in the liver of most mammalian species but does not occur in the pig, making possible the development of a condition similar to gout in this animal in which guanine rather than urate precipitates in the joints [17]. In this report we show that guanase does not occur in circulating red blood cells (both erythrocytes and reticulocytes were examined) of rabbit, sheep or man, but is found in mouse and rat red blood cells. The guanase activity in rat erythrocytes was 15 times greater than in mouse red blood cells. More recently, liver and brain guanase has been shown to have a complicated intracellular distribution [18], to be inducible by guanine administration [19] and to have a proteinaceous guanase inhibitor [19,20]. Rat liver contains two guanase isozymes, whereas only one enzyme is detectable in mouse liver [18]. One rat liver isozyme (the first to elute from DEAE-cellulose) showed a sigmoid dependence on substrate concentration, was activated by GTP and inhibited by allantoin. The other rat liver isozyme had a hyperbolic saturation curve and was not influenced by GTP or allantoin. The single mouse liver guanase was not influenced by GTP or allantoin [21]. In rat reticulocytes two isozymes were detected by DEAE cellulose chromatography. The first one showed classical Michaelis—Menten kinetics and was not influenced by GTP or allantoin. The second isozyme was too unstable to permit a complete kinetic analysis but it was slightly inhibited by allantoin. The data are at present too complicated to allow the deduction of the mechanism by which guanase regulates guanine metabolism in different organs and species. The problem will probably not be solved until the enzyme is purified and the subunit structure of guanase is known. Furthermore, the intriguing question of why there is substantial guanase activity in rat and mouse red blood cells and why this enzyme is absent in the human and other red blood cells that we examined remains to be answered. Recently a guanase has been purified to homogeneity from rabbit liver [22]. The purified enzyme is a single polypeptide chain with a molecular weight of 55 000.

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