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5-METHYLTHIORIBOSE 1-PHOSPHATE: A PRODUCT OF PARTIALLY PURIFIED, RAT LIVER 5'-METHYLTHIOADENOSINE PHOSPHORYLASE ACTIVITY

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Summary

5'-Methylthioadenosine phosphorylase from rat liver has been purified 112-fold. A molecular weight of 90 000 for the enzyme was estimated from gel filtration on Sephadex G-150. The K_m for 5'-methylthioadenosine was $4.7 \cdot 10^{-7}$ M, while the K_m for phosphate was $2 \cdot 10^{-4}$ M. The products of the reaction were isolated and identified as adenine and 5-methylthioribose 1-phosphate. In addition to 5'-methylthioadenosine the nucleoside analogues 5'-ethylthioadenosine and 5'-*n*-propylthioadenosine also served as substrates for the enzyme. The 7-deaza analogue 5'-methylthiotubercidin was found to be an inhibitor of the reaction, but was inactive as a substrate.

Introduction

5'-Methylthioadenosine is a naturally occurring nucleoside whose relationship to methonine and *S*-adenosylmethionine was first demonstrated by Schlenk and Smith [1]. Subsequent investigations have demonstrated that 5'-methylthioadenosine can by synthesized from *S*-adenosylmethionine by at least five separate biochemical mechanisms [2—8]. In spite of these multiple routes of biosynthesis, this nucleoside has been found to be present in low concentrations in those cells and tissues thus far examined [9,10].

Shapiro and Mather [2] showed that in *Enterobacter aerogenes* the

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glycosidic bond of 5′-methylthioadenosine was cleaved releasing 5′-methylthioribose and adenosine. The nucleosidase responsible for this hydrolytic cleavage of 5′-methylthioadenosine was later characterized in more detail in *Escherichia coli* [11,12]. A similar nucleosidase has been found in rat prostatic tissue where phosphate was an absolute requirement, suggesting that the nucleoside was degraded by a phosphorolytic cleavage [13]. More recently, an enzyme from rat lung was purified 30-fold and the products of 5′-methylthioadenosine metabolism were shown to be adenine and 5′-methylthioribose 1-phosphate [14]. This enzyme also has been shown to be present in human placenta [15] and prostate [16].

Because of the observation that 5′-methylthioadenosine inhibits methyltransferase reactions [17], the restriction endonuclease reaction of *E. coli* B [18], RNA synthesis in salivary glands of *Drosophila melanogaster* [19], and human lymphocyte transformation [20], a detailed characterization of the 5′-methylthioadenosine phosphorylase from rat liver was undertaken.

Materials and Methods

Materials

S-Adenosyl-L-[*Me*-^{14}C]methionine was purchased from New England Nuclear and alkaline phosphatase, Type IX from beef liver, was obtained from the Sigma Chemical Company. 5′-Methylthioadenosine sulfoxide, 5′-[*Me*-^{3}H] methylthioadenosine and 5′-[*Et*-^{3}H]ethylthioadenosine were gifts from Dr. Fritz Schlenk. 5′-Methylthiotubercidin was supplied by Dr. James Coward.

5′-[*Me*-^{14}C]Methylthioadenosine was prepared by the acid hydrolysis of *S*-adenosyl-L-[*Me*-^{14}C]methionine [21]. 5-[*Me*-^{14}C]Methylthioribose was prepared from 5′-[*Me*-^{14}C]methylthioadenosine [22].

Preparation of cell free extracts

Mature female rats of the Sprague-Dawley strain were maintained on a diet of Purina rat chow and water ad libitum. 15 female rats were killed by decapitation and exsanguinated. The inferior vena cava was cannulated and the liver perfused with cold 0.3 M sucrose. The liver was removed, minced and suspended in the same solution. The liver sections were then drained and forced through a tissue press. This suspension was then homogenized in 400 ml of 0.5 M sodium potassium phosphate (pH 7.2), 0.1 mM dithiothreitol with a Bellco glass mortar and Teflon pestle and centrifuged at 27 000 × *g* for 45 min in a Sorvall centrifuge. The resulting supernatant fluid was decanted and centrifuged at 100 000 × *g* for 60 min in a Spinco ultracentrifuge; the supernatant fluid from this procedure was stored at 4 °C.

Acetone fractionation

To the 100 000 × *g* supernatant fluid, acetone (A.C.S. grade) was added to 40% saturation. After stirring for 10 min, the precipitate was removed by centrifugation at 27 000 × *g*. The pellet was discarded and the supernatant fluid was brought to 60% saturation with acetone. The precipitate was collected by centrifugation as described above and dissolved in 55 ml of 5 mM potassium phosphate (pH 7.5), 0.5 mM dithiothreitol (buffer 1).

DEAE-Cellulose chromatography

The 40—60% acetone fraction was adsorbed on to a DEAE-cellulose column (2.5 × 20 cm), equilibrated with buffer 1. The column was flushed with buffer 1 and then eluted with a linear 0—300 mM $(NH_4)_2SO_4$ gradient. The fractions which contained enzyme activity were pooled and precipitated with 60% $(NH_4)_2SO_4$, collected by centrifugation and resuspended in 3 ml of buffer 1.

Sephadex G-150 chromatography

The resuspended DEAE-cellulose fractions were applied to a Sephadex G-150 column (2.5 × 40 cm) equilibrated in buffer 1. Proteins were eluted with 600 ml of the same buffer and the fractions containing 5'-methylthioadenosine phosphorylase activity were pooled.

Molecular weight determination

The molecular weight of the enzyme was determined by ascending gel filtration on a Sephadex G-150 column (2.5 × 52 cm) which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.5), 0.1 M NaCl. The column was calibrated with aldolase, ovalbumin, chymotrypsinogen and ribonuclease A. A 2 ml aliquot of the partially purified enzyme was applied to this column and eluted with the equilibration buffer.

Assay for 5'-methylthioadenosine phosphorylase

5'-Methylthioadenosine phosphorylase activity was determined by measuring the conversion of 5'-[*Me*-^{14}C]methylthioadenosine to 5-[*Me*-^{14}C]methylthioribose [12]. The assay mixture contained 0.25 M sodium Hepes, (pH 7.5), 25 mM potassium phosphate (pH 7.5), 0.3 mM 5'-[*Me*-^{14}C]methylthioadenosine ($1.1 \cdot 10^7$ cpm/μmol) and enzyme in a total volume of 0.5 ml. After incubation at 37° C for 20 min the reaction was stopped by the addition of 0.1 ml 1.8 M trichloroacetic acid. The resulting precipitate was removed by centrifugation and 0.2 ml aliquot of the supernatant fluid applied to a Dowex 50 H^+ × 4, 100-200 mesh, column (1.0 × 2.5 cm). Elution of the sample with 3 ml H_2O allowed for the separation of substrate from product. The amount of radioactivity in the eluate was determined in a Beckman LS 250 scintillation spectrometer with 0.4% PPO (2,5-diphenyl-oxazole) in toluene-Triton X-100 (2 : 1). Activity is expressed as nmol 5'-methylthioadenosine converted to 5-methylthioribose/mg protein per incubation period. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol product in 20 min.

Kinetic studies

The standard assay mixture contained 5-[*Me*-^{14}C]methylthioadenosine ($1.1 \cdot 10^8$ cpm/μmol) for determination of the K_m for 5'-methylthioadenosine. The incubation time was varied in separate experiments to maintain 20% conversion of the substrate to product. The reaction was stopped by the addition of 1.8 M trichloroacetic acid and after centrifugation a 0.4-ml aliquot was applied to each Dowex column for elution.

For the phosphate K_m, the partially purified enzyme was concentrated with solid ammonium sulfate and dialyzed against 10 mM sodium Hepes (pH 7.5),

1 mM dithiothreitol to remove any contaminating phosphate. The standard assay mixture contained phosphate concentrations varying from 0.25—1.2 mM. The reaction was incubated at 37° C for 10 min, stopped by acid precipitation and a 0.4-ml aliquot was applied to each Dowex column and eluted with water as described above.

Paper chromatography

Ascending chromatography was performed with Whatman No. 1 paper. The Solvent systems used were: (I) ethanol/water/acetic acid (65 : 34 : 1, v/v) and (II) butanol/water/acetic acid (13 : 5 : 2, v/v). Ultraviolet-absorbing substances were detected with a Mineralight lamp. Sulfur-containing compounds were observed by spraying the chromatograms with potassium iodoplatinate [23], reducing sugars were located with alkaline $AgNO_3$ [24] and phosphoric esters were detected with an acid molybdate reagent [25]. Radioscans of paper chromatograms were run with a Packard Radiochromatogram Scanner.

Product isolation and characterization

The standard assay mixture volume was reduced to 0.4 ml and incubated for 1.5 h. The reaction was stopped by the addition of 2 vol. ethanol and the precipitate removed by centrifugation. A 0.5-ml aliquot of the supernatant fluid was applied to Whatman No. 1 paper and the remaining volume streaked on the same paper. After development in solvent system II and radioscanning of the marker strip, the area corresponding to the migration of the unidentified product above the streaked region was eluted with water. The isolated product was rechromatographed in solvent systems I and II.

Alkaline phosphatase treatment

The isolated Compound X was incubated with alkaline phosphatase (0.14 units) in 0.25 M Tris-HCl, pH 8.0, for 5 min at 37° C. The reaction was stopped with the addition of ethanol. The products of the reaction were examined by paper chromatography in solvent system I.

Substrate specificity

The standard assay mixture contained equal concentrations of 5′-[$^3H_3$]ethylthioadenosine ($2.8 \cdot 10^5$ cmp/μmol) or 5′-[$^3H_3$]methylthioadenosine ($6.2 \cdot 10^5$ cpm/μmol). The concentration of partially purified enzyme was varied over several reactions. A 0.4-ml aliquot was applied to each Dowex column after precipitation of the protein. The respective radioactive sugars were eluted with water.

Protein determination

Protein was determined by the method of Lowry et al. [26]. Samples were monitored on a Gilford Micro-sample spectrophotometer.

Results

Purification of 5′-methylthioadenosine phosphorylase

The purification of 5′-methylthioadenosine phosphorylase is summarized in

Table I. 100 000 × *g* centrifugation of the 30 000 × *g* supernatant fluid may have removed inhibitory substances as the percent yield increased over that seen in the previous step.

Fig. 1 shows the further purification of the acetone fraction using DEAE-cellulose chromatography which resulted in a 52.4 purification (Table I). Elution of the enzyme from a Sephadex G-150 column following DEAE-cellulose chromatography resulted in a purification of approx. 112-fold (Table I) and 6—8 protein bands on analytical polyacrylamide gel electrophoresis. 0.5% bovine serum albumin was added to all buffers to maintain the stability of the purified enzyme preparation.

Molecular weight

The enzyme was eluted from a Sephadex G-150 column at an apparent molecular weight of 90 000 (relative to aldolase, 158 000 daltons; ovalbumin, 45 000 daltons; chymotrypsinogen, 25 000 daltons, and ribonuclease A, 13 700 daltons).

Kinetic constants

The apparent K_m of the partially purified 5′-methylthioadenosine phosphorylase for 5′-methylthioadenosine was found to be $4.7 \cdot 10^{-7}$ M. As the enzymic degradation of 5′-methylthioadenosine by 5′-methylthioadenosine phosphorylase was found to be P_i-dependent, the K_m for phosphate was calculated as 0.2 mM.

Inhibition studies

The inhibitory effects of various compounds on the phosphorylation of 5′-methylthioadenosine are presented in Table II. The nucleoside analogs, 5′-ethylthioadenosine and 5′-*n*-propylthioadenosine, gave more than 50% inhibition at approximately equimolar concentrations. The 7-deaza analog, 5′-methylthiotubercidin, gave 50% inhibition at 7 times the substrate concentration. Other substrate analogs, 5′-dimethylthioadenosine and *S*-adenosyl-L-homocysteine, demonstrated smaller inhibitory effects. Adenine, a product of the reaction, demonstrated high inhibition at both concentrations tested.

TABLE I

ENZYME PURIFICATION

Fraction *	Total protein (mg)	Total units	Specific activity **	Purification (-fold)	Yield (%)
Low speed spin supernatant fluid	21 000	87 570	4.17	1.0	100
Ultracentrifuged supernatant fluid	16 560	110 124	6.65	1.6	125
Acetone fractionation (40—60%)	2 145	68 619	32.0	7.7	78
DEAE-cellulose chromatography	140	31 163	218.32	52.4	36
Sephadex G-150 chromatography	23	10 963	467.91	112.2	13

* The liver was obtained from 15 mature intact female rats.

** Results are expressed as nmol 5′-[*me*-^{14}C]methylthioadenosine converted to product/mg protein per 20 min incubation.

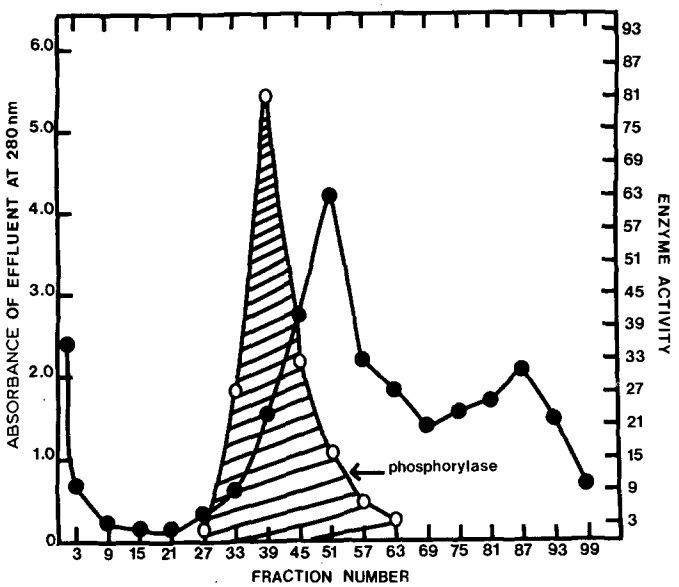

Fig. 1. Elution pattern of 5'-methylthioadenosine phosphorylase from a DEAE-cellulose column Flow rate 0.6—0.7 ml/min; 4.7 ml fractions. ●———●, A_{280}; ○———○, enzyme activity.

The lack of additional inhibition upon raising the concentration of 5'-ethylthioadenosine and 5'-*n*-propylthioadenosine to 30 μM apparently was due to the hydrolysis of these compounds during the course of the reaction.

Substrate specificity

Paper chromatography separation of the products of the reaction using 5'-methylthiotubercidin, 5'-ethylthioadenosine and 5'-*n*-propylthioadenosine as substrates revealed that, whereas the adenine moiety of 5'-ethylthioadenosine and 5'-*n*-propylthioadenosine was one of the products, no purine was formed when 5'-methylthiotubercidin was utilized as substrate. Additional results (not shown) revealed that at 3 different enzyme concentrations 5'-[*Me*-^{3}H]methylthioadenosine was cleaved at 67% the rate of 5'-[*Et*-^{3}H]ethylthioadenosine.

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON 5'-METHYLTHIOADENOSINE PHOSPHORYLASE ACTIVITY *,**

Addition	6 μM (% inhibition)	30 μM (% inhibition)
5'-Methylthiotubercidin	14.3	50.3
5'-Dimethylthioadenosine	10.0	15.2
5'Ethylthioadenosine	58.6	50.0
5'-*n*-Propylthioadenosine	50.2	44.0
S-Adenosylhomocysteine	0.7	17.5
Adenine	43.3	73.7

* 4.4 μM 5'-[*Me*-^{14}C]methylthioadenosine.

** The 112-fold purified preparation served as the enzyme source.

Product identification

The products from a reaction using 5′-[*Me*-^{14}C]methylthioadenosine as substrate and the crude liver enzyme preparation or the partially purified enzyme are shown in Fig. 2. When the crude enzyme source was used, the reaction produced a radioactive compound which migrated at an R_F of 0.82 in solvent system I (Fig. 2B) which corresponded to the migration of 5-methylthioribose in the same solvent system. Using the partially purified enzyme, however, the formation of a radioactive compound which migrated at an R_F of 0.53 was found (Fig. 2C). Respotting this reaction mixture in solvent system II, this same compound (designated Compound X) migrated to an R_F of 0.15 (Fig. 2D) suggesting that Compound X was a product of the reaction and not simply the oxidized form of the substrate, 5′-methylthioadenosine sulfoxide, which migrates to the same area as Compound X in solvent system I.

Compound X was found to be a sulfur- and phosphate-containing sugar by acid-molybdate and iodoplatinate tests. Treatment of Compound X with alkaline phosphatase produced a compound which migrated at an R_F identical with 5-methylthioribose in solvent system I. Negative results were obtained when Compound X was initially sprayed with $AgNO_3$. After alkaline phosphatase treatment, the $AgNO_3$ test was positive suggesting that the reducing position of the sugar in Compound X before phosphatase treatment was blocked.

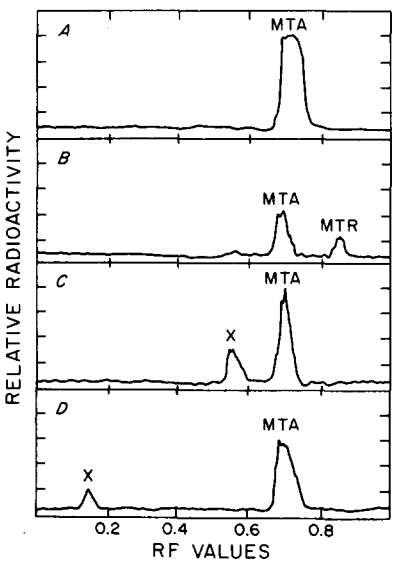

Fig. 2. Radioactivity profiles of paper chromatograms containing the products of the 5′-methylthioadenosine phosphorylase reaction. The distribution of radioactivity on the chromatograms represents (A) reaction mixture minus enzyme, (b) reaction mixture with crude liver enzyme, (C) reaction mixture with partially purified enzyme run in solvent system I. The reaction mixture with partially purified enzyme run in solvent system II (D) is also shown. In all cases 0.3 mm 5′-[*Me*-^{14}C]methylthioadenosine (MTA) was used in the reaction mixture.

Discussion

The data demonstrate the occurrence in rat liver of an enzyme catalyzing the phosphorolytic cleavage of 5′-methylthioadenosine into adenine and 5-methylthioribose 1-phosphate. From the 112-fold purified 5′-methylthioadenosine phosphorylase an apparent molecular weight of 90 000 was calculated. This is similar to the molecular weight of 95 000 for the phosphorylase from purified human placenta [15]. Ferro et al. [12] reported a molecular weight of 31 000 for the nucleosidase purified from *E. coli*. Cacciapuoti et al. [15] have postulated the possible association of three monomers of molecular weight similar to the bacterial enzyme to comprise the mammalian phosphorylase.

An apparent K_m value for 5′-methylthioadenosine of $4.7 \cdot 10^{-7}$ M was determined for the purified rat liver enzyme. This is similar to the $3.0 \cdot 10^{-7}$ M value obtained from the 220-fold purified *E. coli* enzyme [12]. These investigators suggested that the low K_m value of the nucleosidase was responsible for maintaining the low levels of 5′-methylthioadenosine found in *E. coli*. The low levels of this nucleoside in mammalian tissues, therefore, could be attributed to the high affinity that the phosphorylase has for its substrate. Garbers [14] suggested that rat lung 5′-methylthioadenosine phosphorylase catalyzes an equilibrium-ordered reaction and that, if the function of this enzyme is to maintain low levels of the nucleoside, this mechanism would offer an advantage, in that the Michealis constant for 5′-methylhioadenosine would be very low at physiological phosphate concentrations.

The rat liver phosphorylase is activated by P_i, similar to rat ventral prostate [13], rat lung [14], and human placenta enzymes [15], but unlike the bacterial enzyme [12]. The partially purified rat liver enzyme also was found to be inhibited by 5′-methylthiotubercidin, 5′-ethylthioadenosine, 5′-*n*-propylthioadenosine and adenine. At approximately equimolar concentrations to the substrate, 5′-ethylthioadenosine, 5′-*n*-propylthioadenosine and adenine significantly inhibited 5′-methylthioadenosine phosphorylase activity. 5′-Methylthiotubercidin has previously been shown to be a competitive inhibitor of the phosphorylase, but to be inactive as a substrate [27]; our results confirm that data. 5′-Ethylthioadenosine and 5′-*n*-propylthioadenosine have previously been shown to competitively inhibit the nucleosidase of *E. coli* and to also serve as substrates for the enzyme [12]. The data presented here also demonstrate that these two analogs of 5′-methylthioadenosine may serve as substrates for the rat liver 5′-methylthioadenosine phosphorylase.

The observations that 5′-methylthioadenosine is deleterious to cells [17—20], supports the findings that this nucleoside is rapidly degraded and normally not allowed to accumulate. The adenine produced is available to re-enter the pool of adenine nucleotides, whereas the fate of 5-methylthioribose 1-phosphate is unclear. Recently, Ferro et al. [28] demonstrated the existence of an enzyme in *E. aerogenes* that catalyzes the ATP-dependent phosphorylation of 5-methylthioribose. Evidence was presented to indicate that the 5-methylthioribose 1-phosphate synthesized was somehow recycled back into methionine. The fate of 5-methylthioribose 1-phosphate in mammalian tissue has not been elucidated.

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