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KINETIC PROPERTIES AND THE EFFECT OF SUBSTRATE ANALOGUES ON 5'-METHYLTHIOADENOSINE NUCLEOSIDASE FROM *ESCHERICHIA COLI*

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Summary

5'-Methylthioadenosine nucleosidase (EC 3.2.2.-) from *Escherichia coli* has been purified 220-fold. A molecular weight of 31 000 for the enzyme was estimated from gel filtration on Sephadex G-150. The K_m for 5'-methylthioadenosine was $3.1 \cdot 10^{-7}$ M. In addition to 5'-methylthioadenosine, the nucleoside analogues 5'-ethylthioadenosine, 5'-*n*-propylthioadenosine, and *S*-adenosylhomocysteine also served as substrates for the enzyme. These substrate analogues acted as competitive inhibitors of the reaction with 5'-methylthioadenosine. The K_i values for 5'-ethylthioadenosine, 5'-*n*-propylthioadenosine, and *S*-adenosylhomocysteine were determined to be $1.3 \cdot 10^{-7}$ M, $4.6 \cdot 10^{-8}$ M, and $1.92 \cdot 10^{-7}$ M respectively.

Introduction

The observation that 5'-methylthioadenosine inhibits some, if not all, *S*-adenosylmethionine-dependent methyltransferases [1] has stimulated interest in the mechani n by which this compound is metabolized. 5'-Methylthioadenosine has been shown to be synthesized by at least four independent pathways, all originating with *S*-adenosylmethionine [2—12].

In spite of these multiple routes of biosynthesis, 5'-methylthioadenosine has been shown to be present in only trace amounts in a variety of cells and tissues [13,14]. Rhodes and Williams-Ashman [13] found that the concentration of 5'-methylthioadenosine in the ventral prostrate of adult rats is less than 0.2 μmol/g, whereas both spermine and spermidine were found at 5—7 μmol/g in the same tissue. In *Escherichia coli*, the spermidine concentration is between 4.6—7.4 μmol/g dry wt. [15,16], while the 5'-methylthioadenosine level is only 0.38 μmol/g dry wt. [14]. It should be noted that one mol of 5'-methylthioadenosine is produced per mol of spermidine and two mol of 5'-methyl-

thioadenosine are produced per mol of spermine. Any accumulation of this nucleoside would probably be deleterious to cells, not only because of its adverse effect on transmethylations, but because it would also deplete the adenine nucleotide pool. 5′-Methylthioadenosine, however, does not normally accumulate; rather it has been shown to be enzymatically degraded to 5-methylthioribose and adenine in a variety of organisms [2,13,17—19]. The enzyme catalyzing this reaction is 5′-methylthioadenosine nucleosidase (EC 3.2.2.-). Although the metabolic fate of 5-methylthioribose remains unknown, the adenine produced is available to reenter the pool of adenine nucleotides. In mammals, the nucleosidase may account for the repeated demonstration of small quantities of adenine in urine [20]. This enzyme, therefore, plays an important role in the salvage and recycling of adenine.

In addition 5′-methylthioadenosine nucleosidase has been shown to cleave *S*-adenosylhomocysteine to ribosylhomocysteine and adenine [17]. Not only is *S*-adenosylhomocysteine a product of all *S*-adenosylmethionine-dependent methyltransferases, it is also the only other known naturally occurring inhibitor of these methyltransferases [1]. Because of the importance of 5′-methylthioadenosine and *S*-adenosylhomocysteine in cellular metabolism, a detailed characterization of the first enzyme of their catabolism, 5′-methylthioadenosine nucleosidase, was undertaken.

Materials and Methods

Compounds and analyses

[5′-^{14}C]Methylthioadenosine was prepared from *S*-adenosyl-L-[^{14}C]methionine [21]. 5-Methylthioribose was obtained by the acid hydrolysis of 5′-methylthioadenosine [5]. *S*-Adenosyl-L-[^{14}C]methionine was purchased from Amersham Searle, and *S*-adenosylhomocysteine was obtained from Boehringer Mannheim. Hydroxyapatite, Bio-Gel HTP, was purchased from Bio Rad Laboratories. 5′-*n*-Propylthioadenosine, 5′-ethylthioadenosine, and 5′-methylthioinosine were gifts from Dr. Fritz Schlenk.

For paper chromatography, Whatman No. 1 sheets were used for ascending migration in the following solvent systems: ethanol/water/acetic acid (65 : 34 : 1, v/v/v) or *n*-butanol/water/acetic acid (12 : 5 : 3, v/v/v). Ultraviolet absorbing substances were detected with a Mineralight lamp and sulfur-containing compounds were observed by spraying the chromatograms with potassium iodoplatinate [22].

Enzyme purification and assays

The purification of the enzyme is summarized in Table I. Analytical polyacrylamide gel electrophoresis of the purified preparation, performed as described by Brewer and Ashworth [23], revealed one major and several minor bands. 5′-Methylthioadenosine nucleosidase activity was routinely assayed by measuring the formation of [5-^{14}C]methylthioribose from [5′-^{14}C]methylthioadenosine, using ion exchange chromatography to separate the two compounds. The assay mixture contained, unless stated otherwise, 0.05 M potassium phosphate (pH 6.8), 3.2 μM [5′-^{14}C]methylthioadenosine ($1.15 \cdot 10^5$ cpm/μmol), and enzyme in a total volume of 0.5 ml. The reaction mixture was

TABLE I

ENZYME PURIFICATION

The enzyme was purified from a frozen cell paste of *E. coli* B, obtained from Miles Laboratories according to the method of Duerre [17] with the following modifications. The 40—61% ammonium sulfate fraction was dialyzed against 5 mM potassium phosphate buffer (pH 7.0) and applied to a hydroxyapatite column (4.5 X 21 cm) equilibrated with the same buffer. After washing with 1 l of 0.01 M potassium phosphate (pH 7.0) the enzyme was eluted with 1 l of 0.02 M potassium phosphate buffer (pH 7.0). Following DEAE Sephadex A-50 chromatography [17], the enzyme was precipitated with 70% ammonium sulfate. The precipitate was washed successively with 5-ml portions of 60, 55, 50 45, and 40% saturated solutions of ammonium sulfate, pH 7.0. The 45 and 40% extracts contained most of the enzymatic activity, with the highest specific activity in the 45% extract.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)
1. Crude Extract	18 720	848	0.045	1.0
2. $(NH_4)_2SO_4$ Fractionation: (40—61%)	8 522	691	0.081	1.8
3. Hydroxyapatite Chromatography	316	336	1.06	24
4. DEAE-Sephadex A-50 chromatography	22.8	90	3.94	88
5. $(NH_4)_2SO_4$ back extraction: 45% fraction	5.54	55	9.93	220

equilibrated at 37°C and the reaction initiated by the addition of enzyme. At the end of a 10 min incubation period the reaction was terminated by the addition of 0.1 ml 1.8 M trichloroacetic acid. Precipitated protein was removed by centrifugation, and a 0.2 ml aliquot of the supernatant was placed on a Dowex 50 H^+ column (0.5 X 3 cm) X4, 100—200 mesh. Application of the sample was followed by elution of the [5-^{14}C]methylthioribose with 3.0 ml H_2O directly into scintillation vials. Under these conditions [5′-^{14}C]methylthioadenosine is quantitatively retained on the column. Scintillation fluid consisting of 0.4% PPO in toluene/Triton X-100 (2 : 1, v/v) was added to fill the vials and the amount of radioactivity determined in a Beckman LS 250 scintillation spectrometer. One unit of enzyme activity is defined as the amount of enzyme which catalyzed the formation of 1 μmol 5-methylthioribose in 1 min. Throughout the kinetic studies, the incubation time was adjusted so that no more than 10% of the substrate was converted to 5-methylthioribose.

5′-Methylthioadenosine nucleosidase activity was also assayed utilizing unlabelled 5′-methylthioadenosine as the substrate and measuring the amount of reducing sugar, 5-methylthioribose, by the method of Dygert et al. [24]. 5-Methylthioribose, obtained by acid hydrolysis [5], was used as the reference standard, and the lowest level detectable was 0.006 μmol.

Molecular weight determination

The molecular weight of the enzyme was determined by gel-filtration on a Sephadex G-150 column (2.5 X 50 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.8) which contained 0.1 M NaCl and 5 mM β-mercapto-

ethanol [25]. The column was calibrated with aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A.

Protein determination

Protein was determined by the method of Groves et al. [26] using bovine serum albumin as the standard, or by the method of Warburg and Christian [27].

Results

Stability and buffer effects

Solutions of the enzyme stored at −20°C at a protein concentration of 5.0 mg/ml were repeatedly frozen and thawed without loss of activity over a 9 month period. However, upon storage at 4°C only 50% of the enzyme activity was retained after 3 weeks. The enzyme was extremely sensitive to dilution, exhibiting an increasing loss of activity with increasing dilution. The presence of either the substrate or salts, such as K_2SO_4 or KCl, in the dilution buffer did not stabilize the enzyme. However, 20% glycerol stabilized the enzyme to some extent, and 0.5% bovine serum albumin eliminated the loss of activity upon dilution. Therefore, the enzyme was routinely diluted into 0.5% albumin in 0.1 M potassium phosphate (pH 6.8) for all activity assays and kinetic experiments. Enzymatic activity was also affected by the type of buffer in the reaction mixture and dilution medium. Maximal activity was obtained when the buffer in both the assay mixture and dilution medium was potassium phosphate (pH 6.8). In the presence of Tris or HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), enzymatic activity was inhibited 75 and 30%, respectively. The addition of phosphate to the Tris- or HEPES-buffered system did not increase enzyme activity, indicating that the cleavage of 5′-methylthioadenosine was not dependent on the presence of phosphate ions.

Molecular weight

The molecular weight of the enzyme was estimated from gel filtration on a Sephadex G-150 column, using proteins of known molecular weight as standards. The 5′-methylthioadenosine nucleosidase was estimated to have a molecular weight of approximately 31 000.

Kinetic constants and substrate specificity

The reaction velocity of 5′-methylthioadenosine nucleosidase showed normal Michaelis-Menten kinetics with 5′-methylthioadenosine as the variable substrate. An apparent K_m value for 5′-methylthioadenosine of $3.1 \cdot 10^{-7}$ M was calculated from a double reciprocal plot.

In addition to 5′-methylthioadenosine, the enzyme also showed hydrolytic activity toward the nucleosides 5′-*n*-propylthioadenosine, 5′-ethylthioadenosine, and *S*-adenosylhomocysteine as shown in Table II. The rates of the reactions were determined by measuring the production of a reducing sugar. In a separate experiment the products of the reaction with 5′-*n*-propylthioadenosine and 5′-ethylthioadenosine as substrates were examined by paper chromatography. In both cases adenine was one of the products, and the other products

TABLE II

RELATIVE RATES OF HYDROLYSIS OF VARIOUS NUCLEOSIDES BY 5'-METHYLTHIOADENOSINE NUCLEOSIDASE

The rate of hydrolysis of 5'-methylthioadenosine was taken as 100%. The rates were determined by measuring the production of reducing sugars as described in Materials and Methods. All nucleosides were present at a final concentration of 3.0 mM.

Substrate	Relative activity (%)
5'-Methylthioadenosine	100
5'-Ethylthioadenosine	118
5'-*n*-Propylthioadenosine	106
S-Adenosylhomocysteine	35

were the respective sugars, 5-*n*-propylthioribose and 5-ethylthioribose. The thio sugars were identified using acid hydrolyzates of 5'-ethylthioadenosine and 5'-*n*-propylthioadenosine as standards and detecting the sulfur-containing sugars with potassium iodoplatinate spray. The products of the reaction with *S*-adenosylhomocysteine as the substrate were adenine and ribosylhomocysteine, as has been demonstrated previously [17].

Inhibition studies

The inhibitory effects of various compounds on the hydrolysis of 5'-methylthioadenosine are presented in Table III. The nucleoside analogues 5'-ethylthioadenosine, 5'-*n*-propylthioadenosine, and *S*-adenosylhomocysteine, which also serve as substrates in the reaction, exhibit the greatest degree of inhibition. 5'-Methylthioinosine, which is also a substrate analogue but is not hydrolyzed by 5'-methylthiodenosine nucleosidase, does not significantly inhibit the reaction.

TABLE III

INHIBITORY EFFECTS OF VARIOUS COMPOUNDS ON 5'-METHYLTHIOADENOSINE NUCLEOSIDASE ACTIVITY

5'-Methylthioadenosine nucleosidase activity was measured as described in Materials and Methods using [5'-^{14}C]methylthioadenosine as the substrate at a concentration of 3.2 μM. All other compounds tested were present at a final concentration of 0.3 mM.

Addition	Activity (μmol/min)	% inhibition
None	36.3	—
5'-*n*-Propylthioadenosine	0.16	99.5
5'-Ethylthioadenosine	0.31	99.2
S-adenosylhomocysteine	0.53	98.6
Adenine	25.2	30.6
5'-Methylthioinosine	29.3	19.3
L-methionine	31.4	13.5
AMP	31.5	13.2
5-Methylthioribose	34.8	4.1
Putrescine	35.5	2.4
Spermidine	35.6	2.1

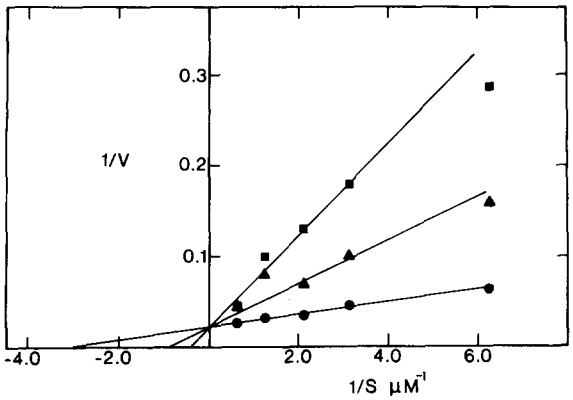

Fig. 1. Double-reciprocal plot of initial reaction velocity versus 5′-methylthioadenosine concentration in the absence and in the presence of two fixed concentrations of 5′-ethylthioadenosine. ●———●, zero; ▲———▲, 0.38 μM; ■———■, 0.75 μM.

The degree of inhibition indicated for adenine is probably not significant since adenine was present in the reaction at a concentration one hundred times that of the substrate. The effects of putrescine and spermidine were investigated because of their involvement in one of the major pathways of 5′-methylthioadenosine production. These compounds, however, apparently do not have a regulatory effect on the 5′-methylthioadenosine nucleosidase reaction.

The results of a kinetic analysis of the inhibition by the nucleoside analogue 5′-ethylthioadenosine is shown in Fig. 1. Similar experiments were carried out with 5′-*n*-propylthioadenosine and *S*-adenosylhomocysteine. The inhibition pattern of all three compounds was competitive and the K_i values for 5′-ethylthioadenosine, 5′-*n*-propylthioadenosine, and *S*-adenosylhomocysteine were found to be $1.3 \cdot 10^{-7}$ M, $4.6 \cdot 10^{-8}$ M, and $1.92 \cdot 10^{-7}$ M, respectively.

Discussion

These data demonstrate that significant differences exist between the 5′-methylthioadenosine nucleosidase utilized in the present investigation and the corresponding enzymes examined by Duerre [17] and Pegg and Williams-Ashman [19]. In this study, the 5′-methylthioadenosine nucleosidase was purified from a soluble fraction and has a molecular weight of approximately 31 000 as determined by filtration on a Sephadex G-150 column. The products of the enzymatic reaction were identified as 5-methylthioribose and adenine. Unlike the rat prostate enzyme [19], the nucleosidase from *E. coli* was not dependent on phosphate ions, indicating that cleavage of the glycosyl linkage is of the hydrolytic rather than of the phosphorolytic type. This is in agreement with the results obtained by Duerre [18]. We have, however, found that maximal activity of the enzyme is obtained when the enzyme is diluted and assayed in potassium phosphate buffer.

Previous kinetic studies on the 5′-methylthioadenosine nucleosidase have revealed an apparent K_m for 5′-methylthioadenosine of $1.8 \cdot 10^{-3}$ M for a 160-

fold purified enzyme from *E. coli* [17], and $3 \cdot 10^{-4}$ M for a 30-fold purified enzyme from the rat ventral prostrate [19]. In the present study, an apparent K_m value for 5′-methylthioadenosine of $3.1 \cdot 10^{-7}$ M was calculated for a 220-fold purified enzyme from *E. coli*. As stated previously the level of 5′-methylthioadenosine in *E. coli* has been determined to be 0.38 μmol/g dry wt. [14]. Assuming that one *E. coli* cell weighs $4 \cdot 10^{-13}$ g (dry wt.) [28] and that the volume of a single cell is $1 \cdot 10^{-12}$ ml [29], then the 5′-methylthioadenosine concentration in *E. coli* is $1.52 \cdot 10^{-4}$ M. The kinetic data from the present experiments indicate that the 5′-methylthioadenosine nucleosidase alone is capable of maintaining the low levels of 5′-methylthioadenosine found in *E. coli* cells.

Besides 5′-methylthioadenosine, three other nucleosides were hydrolyzed by the nucleosidase: 5′-ethylthioadenosine, 5′-*n*-propylthioadenosine, and *S*-adenosylhomocysteine. Catabolism of the latter compound is in agreement with observations of Duerre [17]. 5′-Ethylthioadenosine and 5′-*n*-propylthioadenosine acted as somewhat better substrates than 5′-methylthioadenosine, but *S*-adenosylhomocysteine was cleaved at only 35% the rate of 5′-methylthioadenosine. Duerre [17] has previously shown that the ratio of the rate of cleavage of *S*-adenosylhomocysteine to the rate of cleavage of 5′-methylthioadenosine was 0.60. The discrepancy in these results may be due to the use of ribose as the standard for the measurement of the reducing sugar by Duerre [17] and the use of 5-methylthioribose as the standard in the present investigation.

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