SELECTIVE INHIBITIONS OF tRNA METHYLTRANSFERASES BY S-ADENOSYLHOMOCYSTEINE AND TWO OF ITS ANALOGUES‡

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1. Introduction

S-adenosyl L-homocysteine (SAH)* is a potent inhibitor of nearly all the methyltransferases (EC.2.2.1) tested [1–7]. However, differences in the degree of inhibition by SAH of various methyl transferases present in a same extract have been found [4, 6].

Recently, we reported the synthesis of several compounds, related to SAH [8, 9]. Among them, S-adenosyl L-cysteine and S-adenosyl cysteamine were found to inhibit *E. coli* B tRNA methylation by a N-2-guanine methyltransferase partially purified from rabbit liver [7].

In this communication we report the effect of SAH and of the two synthetic analogues SACysteine and SACysteamine on the pattern of methylation, in a rate reaction experiment of methylation of an unfractionated *E. coli* B tRNA by a rabbit liver methyltransferase extract.

2. Materials and methods

S-adenosyl L-methionine methyl [^{14}C](SAM) was obtained from CEA Saclay: specific activity 50 mCi/mmole; 97% purity. It was diluted and adjusted to pH 4 prior to use. *E. coli* B unfractionated tRNA was obtained from Chagrin Falls, Ohio, USA.

2.1. *Inhibitors*

5′-S-adenosylhomocysteine, 5′-S-adenosylcysteine and 5′-S-adenosylcysteamine were prepared as described earlier [8, 9].

2.2. *Methyltransferases*

The method described by Pegg [10] was followed in order to obtain a purified extract containing all the tRNA methylases. The preparation was unstable and lost over 60% of its activity in a week, at −20°.

2.3. *Enzymatic assays*

The assays contained, in a total volume of 0.5 ml, 50 μmole of Tris-HCl buffer, pH 8.5; 10 nmole of SAM methyl [^{14}C]; 0.2 μmole of 2-mercaptoethanol, 0.4 to 1.6 mg methylase preparation, 10 μg tRNA, ammonium acetate at a concentration of 0.2 moles and additions as described in the text. Ammonium acetate was added because of its known rate and extent enhancing effect on methylation [10, 13], a concentration of 0.2 mole of $NH_4^{\oplus}$ corresponding approximately to the level required for an optimum methylation of tRNA.

Incubation was performed at 37° for 1 hr; then 100 μl portions of each incubation mixture were deposited in duplicate on Whatman filter paper discs no. 3 MM; 2.4 cm diameter. The discs were treated according to the modified Novelli method as described by Pegg [10]. Samples were removed prior to incubation in order to correct for the amount of

Abbreviations:
SAH: S-adenosylhomocysteine.
SAM: S-adenosylmethionine.
SAC: S-adenosyl L-cysteine.
SACysteamine: S-adenosylcysteamine.

‡ This paper is dedicated by E.L. to his friend and colleague Theodor Wieland for his 60th birthday.

radioactivity adsorbed non specifically on the discs. The methylation of proteins was determined by treating one of the series of discs with hot 5% TCA in order to hydrolyse the tRNA [10]. The discs were then air-dried and placed in scintillation vials, covered with 10 ml of toluene scintillation fluid and counted in a Nuclear Chicago Mark II apparatus operating at an efficiency of 82%.

2.4. *Analysis of radioactive bases*

The standard assay medium was scaled up twenty times. Carrier tRNA (4.5 mg) was added to the reaction mixture (10 ml) and the tRNA, extracted by the phenol procedure [14]. The resulting nucleic acid was dissolved in 2.5 ml of hydrochloric acid (1 N) and hydrolysed at 100° for 1 hr.

The hydrolysate was dried at 60° under reduced pressure and 0.5 ml of hydrochloric acid (0.01 N) added. Each sample thus obtained was chromatographed first on Whatman no. 1 paper strips, 3.5 cm wide along with methylated bases as markers. N-2 methylguanine and N-2 dimethylguanine were kindly provided by Dr. A.E. Pegg, Courtauld Institute, Middlesex Hospital, London.

The major radioactive bases were detected by their UV absorbance after chromatography, separately, by a combination of the following three solvent systems: A) n-butanol–water–concentrated ammonia (86:9:5; v/v), B) methanol–water–concentrated hydrochloric acid (7:1:1; v/v), C) n-butanol–water–acetic acid (4:1:1; v/v).

The radioactivity of spots was first determined with a Scanner (Dünnschicht Scanner, Berthold LB 2720) and the spots were then eluted with 0.01 N hydrochloric acid (20 ml). The eluate was dried under reduced pressure redissolved in 0.1 ml hydrochloric acid and rechromatographed in one or more of the above mentioned solvents until homogeneous. Finally the radioactive areas were cut into strips 1 cm wide; each segment was put separately into scintillation vials covered with 15 ml toluene scintillation fluid and counted for 40 min. Segments were cut from areas devoid of radioactivity for blank determinations. Determination of radioactive 5-methylcytosine and 5-methyluracil was performed by perchloric acid treatment of the base line after the first elution in solvent A as described elsewhere [10]. Removal of perchloric acid after hydrolysis of the pyrimidine nucleotides, was carried out at 70° under high vacuum.

Table 1

Inhibition of *E. coli* B tRNA methyltransferases from rabbit liver by S-adenosyl homocysteine (SAH), S-adenosyl L-cysteine (SAC) and S-adenosyl cysteamine (SACysteamine).

Inhibitors	Conc. (μM)	Inhibition (%)
SAH	10	48
	100	68
	1000	80
SAC	10	21
	100	49
	1000	60
SACysteamine	10	16
	100	29
	1000	64

Incubations were performed at 37° for 1 hr and were processed as described above. Each assay contained in a total volume of 0.5 ml, 50 μmoles of Tris buffer, pH 8.5, 10 μmoles of SAM methyl [^{14}C]; 0.2 μmole of 2-mercaptoethanol; 0.4 to 1.6 mg enzymes, 10 μg tRNA ammonium acetate at 0.2 moles concentration and the amounts of inhibitors shown above.

3. Results and discussion

Under the experimental conditions described above, the rate of methylation of tRNA at 37° was found to be proportional to time for at least 60 min.

A preliminary experiment was performed in order to evaluate the inhibitory activity of each of the inhibitors. The results are summarized in table 1.

3.1. *Inhibition by S-adenosylhomocysteine (SAH)*

Examination of the pattern of methylated bases obtained in the control experiment (without SAH) showed that under the conditions used for the assay, the principal methylated bases formed were: 2-methylguanine, 1-methyladenine, and 5-methylcytosine (table 2).

When the mixture of enzymes was incubated in the presence of 10 μmole SAH, an overall inhibition of 41% of the methylation of the tRNA was found (table 2). However, the action of SAH was found to be selective. For instance nearly complete inhibition

Table 2
E. coli B tRNA methylated with rabbit liver enzymes.

Methylated bases detected	Control (cpm)	SAH (cpm)	SAH Inhibition (%)	SAC (cpm)	SAC Inhibition (%)	SAC Corrected** inhibition (%)	SACysteamine (cpm)	SACysteamine Inhibition (%)	SACysteamine Corrected** inhibition (%)
1-Methylguanine	101	118	0	194	0	0	126	0	0
2-Methylguanine	2670	1912	28	2074	22	32	1807	32	57
3-Methylguanine	623	350	44	412	34	49	605	0	0
2-N-dimethyl-guanine	351	33	91	186	47	67	207	41	73
7-Methylguanine	306	312	0	336	0	0	306	0	0
1-Methyladenine	1265	576	54	766	48	57	923	27	48
2-Methyladenine	421	N.D.*	100	127	70	~100	419	0	0
6-Methyladenine	N.D.*	N.D.*	–	N.D.*	–	–	N.D.*	–	–
5-Methylcytidine	1104	769	30	794	28	40	822	34	46
5-Methyluracil	55	N.D.*	–	30	–		73	–	
Overall inhibition			41		29	41	23		41

Analysis of radioactive bases: effect of S-adenosyl homocysteine (SAH), S-adenosyl cysteine (SAC) and S-adenosyl cysteamine (SACysteamine) on the methylation of *E. coli* B tRNA in a rate reaction experiment. Each incubation mixture, contained in a total volume of 10 ml: 200 μg of tRNA 0.2 μmoles of SAM methyl [^{14}C] in Tris buffer pH 8.5; ammonium acetate was present at a concentration of 0.2 moles. After incubation at 37° for 1 hr, the samples were processed as described under Methods; concentration of inhibitors: SAH 10 μM; SAC 100 μM SACysteamine 100 μM.

* N.D. not detected.

** Corrected inhibition values (%): the overall inhibition values obtained in the experiments with SAC and SACysteamine were converted to the overall value of inhibition of SAH (41%). The corresponding conversion factors were then multiplied by the values of inhibition found for each of the methylated bases: i.e. $\frac{\text{overall inhibition with SAH}}{\text{overall inhibition with SAC}} = 1.43 = \text{conversion factor}$. In this table, the values found for 5-methyluracil and 1-methylguanine were omitted.

was obtained with the 2-C-adenine and the 2-di-N guanine methylating enzymes, whereas only 28% and 30% inhibitions were observed, respectively, with the 2-N-adenine and the 5-C-cytosine methylating enzymes. An apparent lack of inhibition of the 7-N-guanine methylating enzyme was noticed. It can be seen that two of the major methylating enzymes (forming 2-methylguanine and 5-methylcytosine) are the least sensitive to the action of SAH. On the other hand minor methylating enzymes such as those producing 2-di-N-methylguanine, and the 2-C-methyladenine seemed to be more sensitive to the action of this inhibitor (inhibitions of 91 and ≃100%, respectively).

The selective inhibitory action of SAH on various partially purified *E. coli* methyltransferases has been described already by Hurwitz et al. [4]. More recently Kerr, [6] has shown that SAH acted more selectively at the same concentration on a tRNA methyltransferase, than on a glycine methyltransferase present in a same extract.

Our *in vitro* experiments suggest that SAH is an especially potent inhibitor of some of the minor methylases present in the cell whereas major methylating enzymes are less sensitive to its action. Such a selective inhibition might be a direct consequence of the affinity of these enzymes for SAH and would depend on the conditions in which methylation occurs (i.e. ionic factors [10, 13, 15, 20, 23], pH of the medium etc...).

It is thus conceivable that the percentage of the various methylated bases is largely dependent on the degree of inhibition by SAH of the corresponding methylases.

The controversial formation of "neo methylases" [16, 18, 21, 22] in transformed tissues or cells could be the result of different environmental conditions leading to a less inhibitory activity of SAH and to a changed pattern of methylation.

3.2. *Inhibition by S-adenosylcysteine (SAC) and S-adenosylcysteamine (SACysteamine)*

The inhibition levels obtained with the analogues of SAH:SAC and SACysteamine are reported in table 2. Concentrations of 100 μM were necessary in order to obtain these levels as compared to 10 μM for SAH. The overall inhibitions obtained are, respectively, 29 and 23% indicating, as mentioned in a previous communication, a weaker inhibitory activity of these two products as compared to SAH [7].

In order to simplify the comparison of the extents of inhibition between the three inhibitors, the overall inhibition values obtained with SAC and SACysteamine were brought to the same overall value as the one obtained for SAH (41%). Accordingly, the value of inhibition observed for the formation of each methylated base in the SAC and the SACysteamine experiments, was corrected by multiplying each of the values by the corresponding factor as indicated in table 2. It can be seen that the inhibitory action of SACysteine is qualitatively similar to the action of SAH showing thus that no fundamental difference exists between these two products concerning their binding to the various methyltransferases. However SACysteamine is unable to inhibit the 2-N-guanine and the 2-C-adenine methylating enzymes, it seems to inhibit more selectively than does SAH the 2-N-guanine and the 5-C-cytosine methylating enzymes.

Recently [19] a "modification enzyme" (a DNA methylation enzyme) was shown to be inert towards the action of SAH, but inhibited by analogues such as 5′-methylthioadenosine or 5′-S-adenosylethionine. Thus in certain cases, the lack of inhibitory activity of the natural inhibitor SAH can be overcome by the use of analogues having different or simpler structures.

It is one of our aims to prepare synthetic analogues of SAH or SAM, which might act in a selective or specific manner within a group of methyltransferases present in a given extract.

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