CYTOKININS THAT INHIBIT TRANSFER RNA METHYLATING ENZYMES

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1. Introduction

Regulation of the tRNA methylases is, as yet, only poorly understood, but alteration in specificities and levels of activities of these enzymes has been observed after virus infection [1], latent virus induction [2, 3], in transformed cells [4], and in neoplasms [5–11]. Naturally occurring inhibitors of the tRNA methylases have been observed after lambda bacteriophage induction [2] and in normal adult animal tissues [12]. Adenosine [13], adenine, and several cytotoxic analogs of these compounds [14] were found to be inhibitors of tRNA methylating enzymes. These original findings have been confirmed and extended by other investigators [15]. In this paper, we report that four adenosine analogs which are cytokinins [16] have been found to be inhibitors of transfer RNA methylating enzymes *in vitro*. These compounds include 6-furfurylamino purine riboside (Kinetin riboside), N^6-(Δ^2-isopentenyl) adenosine, N^6-(trans-4-hydroxy-3-methylbut-2-enyl) adenosine (zeatin riboside), and 6-benzylamino purine riboside. Each of these cytokinins inhibited the rate at which $^{14}CH_3$ transfer from $^{14}CH_3$-S-adenosyl methionine to methyl-deficient tRNA was catalyzed by enzyme extracts from animal and bacterial cells. Kinetin riboside was found to be a competitive inhibitor of the enzyme-catalyzed methyl transfer from S-adenosyl methionine to guanine in tRNA. Partial purification of calf spleen enzymes by precipitation with 60% saturated $(NH_4)_2SO_4$ did not alter the response to inhibitors.

2. Materials and methods

2.1. *Enzyme preparations*

Cells of *E. coli* M3S were grown aerobically at 32° in a medium consisting of 10 g Bactotryptone, 1 g yeast extract, 7 g NaCl and 0.9 g glucose per liter.

When the population density reached 5×10^8 cells/ml, the cells were harvested by centrifugation in the cold and washed with cold saline. Cells of *E. coli* B, harvested in mid-logarithmic growth phase, were purchased, as a frozen paste, from Miles Laboratories, Inc. Cells were disrupted by grinding in the cold with 3 times their weight of wet alumina A-305 and extracted with 0.01 M Tris, 0.01 M $MgCl_2$, 0.005 M mercaptoethanol buffer, pH 8, (SMB) containing 5 μg/ml DNase. The supernatant, after centrifugation at 8000 *g* at 5° for 20 min, was centrifuged at 100,000 *g* for 70 min. This second supernatant contained most of the tRNA methylase activity of the cells.

About 6 g of fresh frozen calf spleen was disrupted by grinding for 2 min at 0° in a Potter-Elvehjem homogenizer in 0,25 M sucrose, 0.015 M $MgCl_2$. The suspension was centrifuged at 10,000 *g* for 20 min at 5° and the supernatant then centrifuged at 100,000 *g* for 1 hr to yield an extract containing tRNA methylase activity.

2.2. *Enzyme assays*

Each assay tube, containing in a vol of 0.3–0.5 ml 75–100 μg methyl deficient tRNA, 0.02–0.2 μCi $^{14}CH_3$-S-adenosyl methionine (specific activity 26–30 mCi/mmole), enzyme extract, and varying concentrations of the cytokinins dissolved in SMB,

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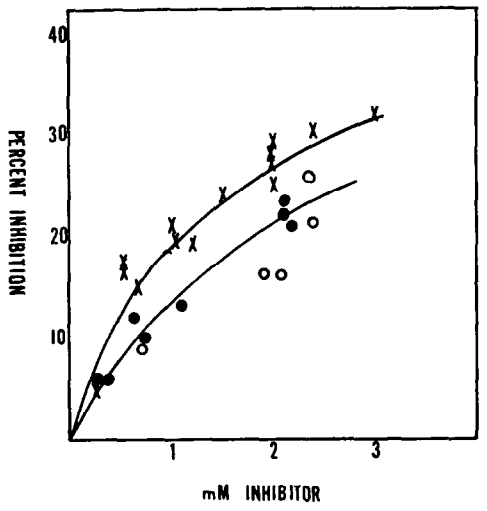

Fig. 1. Inhibition of bacterial tRNA methylases by adenosine riboside (AR), kinetin riboside (KR) and zeatin riboside (ZR). Each incubation tube contained in a vol. of 0.45 ml, 75 μg methyl-deficient tRNA, 0.1 μCi ^{14}C-SAM (specific activity 55 mCi/mmole), inhibitor dissolved in 0.01 M Tris, 0.01 M $MgCl_2$, 0.005 M mercaptoethanol, pH 8, and *E. coli* B enzyme extract (0.1 mg protein) in a final buffer concentration of 0.015 M Tris, 0.015 M $MgCl_2$, 0.0075 M mercaptoethanol, pH 8. After 30 min incubation at 35° the reaction was terminated by addition of hydroxylamine, pH 7.5. ^{14}C transferred to RNA was measured after precipitation and washing of RNA as described above. X = AR, • = KR, ○ = ZR.

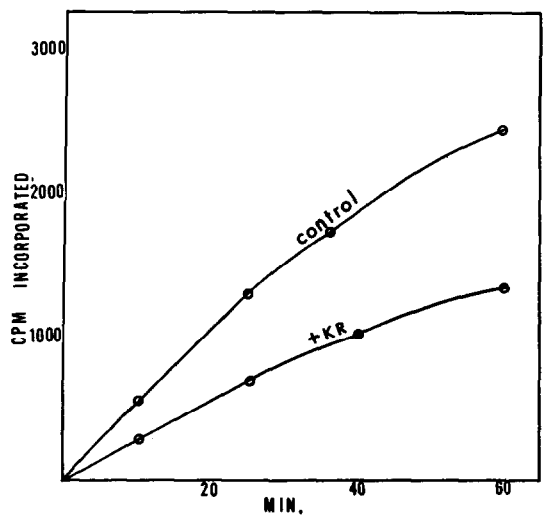

Fig. 2. Inhibition of calf-spleen tRNA methylases by kinetin riboside. Each incubation tube contained, in a volume of 0.43 ml, 75 μg methyl-deficient tRNA, 0.08 μCi ^{14}C-SAM (specific activity 55 mCi/mmole), calf spleen enzyme extract (0.77 mg protein), and 400 μg KR (where used) in a final buffer concentration of 0.015 M Tris, 0.015 M $MgCl_2$, 0.0075 M mercaptoethanol, pH 8. Samples were withdrawn from the incubation bath at various time intervals and processed as described above.

all in a final concentration of 0.015 M Tris, 0.015 M $MgCl_2$, 0.007 M mercaptoethanol, pH 8, was incubated at 35° for 30–90 min. Enzyme levels were kept below one half saturation [1]. 0.2 ml of 1.5 M NH_2OH, pH 7.5, was added to each tube, and the incubation was continued for 10 min longer. Samples were chilled, and 2 mg carrier RNA, followed by 4 ml of 0.5 M NaCl in 75% ethanol were added. After a minimum of at least 2 hr at −15°, the precipitates were collected by centrifugation in the cold. Precipitates were extracted with 2 M NaCl [17] and RNA was reprecipitated from the extracts by addition of 50% TCA solution. These precipitates were washed in the cold with 10% TCA solution, alcohol, and ether. RNA, dissolved in a small volume of 2 M NH_4OH was assayed for radioactivity in a scintillation counter.

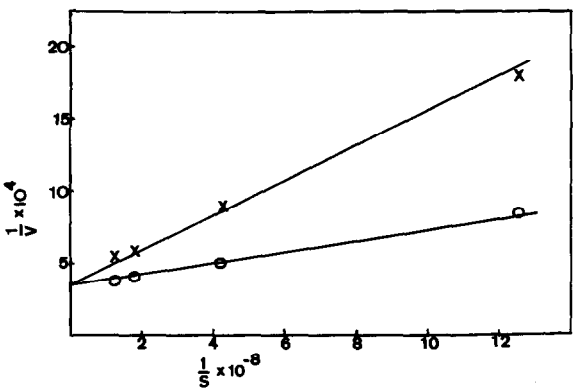

Fig. 3. Competitive inhibition of tRNA methylation by kinetin riboside. 0.45 ml samples containing 75 μg methyl-deficient tRNA, 400 μg kinetin riboside (where used), calf-spleen enzyme preparation (0.77 mg protein), 0.02–0.2 μCi ^{14}C-SAM (specific activity 55 mCi/mmole), in a final buffer concentration of 0.015 M Tris, 0.015 M $MgCl_2$, 0.005 M mercaptoethanol, pH 8, were incubated at 35° for 60 min. Samples were then processed as described above. S = molar conc. of SAM. V = cpm incorporated. ○ = controls. X = samples + KR.

2.3. *Cytokinins*

Isopentenyladenosine was a gift from Dr. N.J. Leonard. Additional samples of this compound were purchased from Sigma Chemical Co. The sources of the other cytokinins were as follows: zeatin riboside, Calbiochem; 6-benzylamino-purine riboside, Sigma Chemical Co., kinetin riboside, Nutritional Biochem. Co.

3. Results

Earlier experiments had shown that isopentenyladenosine (IPA) was an effective inhibitor of tRNA methylases extracted from calf tissues [14]. IPA, a naturally occurring minor component of tRNA, has been reported to exhibit cytokinin activity, cytotoxicity, and antitumor activity [16, 18, 19]. It seemed of interest to investigate the possibility that other cytokinins might also be able to inhibit tRNA methylation.

The effects of kinetin riboside, (6-furfurylamino purine riboside, KR) which is a synthetic cytokinin, and of a naturally occurring cytokinin, zeatin riboside (ZR), with the chemical structure trans-6-(4-hydroxy-3-methyl-but-2-enyl)-aminopurine riboside on tRNA methylases were tested. As shown in fig. 1, KR and ZR inhibited the catalysis, by *E. coli* extracts, of methyl transfer from S-adenosyl-methionine to methyl-deficient tRNA. Adenosine, which was shown earlier to be an inhibitor of tRNA methylases of *E. coli* [13–15] was used here as a reference compound (fig. 1). The effects of KR and of ZR were found to be equivalent on a molar basis, when enzyme concentration and S-adenosyl-methionine concentration were kept constant.

Since earlier experiments had shown that adenine and adenosine preferentially inhibit guanine tRNA methylase, [14] tests were made to determine whether KR inhibits all the tRNA methylases to an equal extent or whether it is selective. *E. coli* preparations contain a mixture of tRNA methylating enzymes in which uracil methylase is the largest component [1, 3, 20]. In the presence of 1.1×10^{-3} M KR, 1.8×10^{-8} M S-adenosyl methionine, and just minimally saturating levels of unfractionated *E. coli* M3S enzymes, methyl-deficient tRNA accepted CH_3 groups into uracil and guanine in the ratio of about 7:1. Control preparations, without KR accepted uracil and guanine in the ratio of 4:1. These data indicated that guanine methylation was more sensitive to KR inhibition than was uracil methylation.

In normal tissue extracts most of the tRNA methylating activity is represented by guanine tRNA methylases [8]. Animal tissues seemed, therefore, to be a more suitable source for enzymes with which to study further the effects of KR on tRNA methylation. Fig. 2 shows the time course of methyl transfer catalyzed by calf-spleen enzymes in the presence and absence of KR. These data indicate that the rate of methyl-group transfer was decreased by KR throughout the period of the incubation. Experiments were then carried out in the presence of 1.5×10^{-3} M KR and varying concentrations of S-adenosyl methionine (SAM). The data shown in fig. 3 indicate a typical competitive inhibitor relationship between KR and the methyl donor, SAM.

Since IPA is not readily soluble in aqueous solutions, control of concentration in earlier experiments was difficult. It seemed worthwhile to reexamine the influence of IPA on bacterial methylases under slightly altered conditions. Addition of 0.1% Tween 80 to the buffer does not interfere with tRNA methylase activity. As shown in table 1, when IPA was solubilized by the addition of Tween 80 detergent, it was an inhibitor of tRNA methylating enzymes from *E. coli.* The detergent was also used in experiments with 6-benzylamino purine riboside. This synthetic cytokinin also caused a decrease in the rate of methylation catalyzed by either animal or bacterial preparations (table 1).

4. Discussion

The studies described here indicate that several N6 substituted analogs of adenosine are inhibitors of tRNA methylating enzymes. Each of the substances used in the current studies has been reported to possess cytokinin activity [16, 19]. It should be noted that there are analogs which have either hydrophobic or hydrophilic substituents at the N6 position in this group. Some of these cytokinins are known to be cytotoxic in animal cells and to possess antitumor activity [18, 21, 22]. In addition, IPA has been shown to affect lymphocyte transformation [23].

Table 1
Inhibition of tRNA methylases by isopentenyladenosine and by 6-benzylamino purine riboside.

Inhibitor	Inhibitor Conc. ($\times 10^{-3}$ M)	Enzyme Source	SAM Conc. ($\times 10^{-8}$ M)	$^{14}CH_3$ Incorp. (cpm)	Inhibition (%)
	0	*E. coli* B	2	8260 ± 340	–
IPA	2.0	*E. coli* B	2	6450 ± 330	22
	0	*E. coli* B	2	7675 ± 220	–
IPA	2.1	*E. coli* B	2	5935 ± 1	23
	0	*E. coli* B	2	7445 ± 145	–
IPA	2.0	*E. coli* B	2	5935 ± 345	19
6BA	1.4	*E. coli* B	2	6145 ± 20	17
	0	*E. coli* B	0.16	1155 ± 25	–
6BA	1.9	*E. coli* B	0.16	930 ± 20	20
	0	calf spleen	0.16	1355 ± 50	–
6BA	0.6	calf spleen	0.16	1070 ± 40	21
	1.3	calf spleen	0.16	935 ± 2	31
	1.9	calf spleen	0.16	797 ± 10	40

See the legends to the figures for incubation conditions.

At least one of the factors involved in control of tRNA methylase activity *in vivo* is the occurrence of varying levels of intracellular methylase inhibitors. Development of a low molecular weight inhibitor of uracil methylase in *E. coli* is associated with lambda prophage induction [2, 20]. The levels of naturally occurring inhibitors of methylases in animal tissues vary with age and hormonal state of the animal [12]. Nicotinamide has also been found to inhibit tRNA methylases [2, 4]. It seems possible that the cytokinins may constitute another class of naturally occurring regulators of tRNA methylating enzymes. However, much additional work will be necessary to determine whether the substances described here perform such a function *in vivo*, or whether their property of inhibiting tRNA methylases is primarily of pharmacologic interest.

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