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Inhibition of bacterial DNA cytosine-5-methyltransferase by *S*-adenosyl-L-homocysteine and some related compounds†

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S-Adenosyl-L-homocysteine and five related compounds have been evaluated as inhibitors of a DNA cytosine-5-methyltransferase. DNA methylation was assayed in cell extracts from *E. coli* strain J6-2 *dcm*$^+$, proficient in DNA cytosine-5-methyltransferase activity, containing substrate DNA isolated from *E. coli* strain J6-2 *dcm*$^-$, a strain deficient in DNA cytosine-5-methyltransferase. *S*-Adenosyl-L-homocysteine and its 7-deaza analogue, *S*-tubercidinylhomocysteine, were competitive inhibitors of DNA cytosine-5-methyltransferase with K_i's of 14·2 and 17·6 μM, respectively, in the above enzyme assay.

It is now well established that one of the functions of prokaryotic DNA methyltransferases is to protect the host DNA against cleavage by indigenous nuclease enzymes, by introducing methyl groups at certain 'recognition sites' along the DNA molecule. The absence of methyl groups at these sites results in cleavage of the DNA by the cells own nucleases. In this respect, we have been interested in the development of potent inhibitors of prokaryotic DNA methyltransferases, as a basis for the design of new antibacterial agents.

Only two naturally occurring methylated bases are generally found in prokaryotic DNA, namely, 5-methylcytosine and N^6-methyladenine (Hattman 1981). These methylated bases usually comprise only a minor fraction of the total DNA bases. The DNA methyltransferases transfer methyl groups from donor *S*-adenosyl-L-methionine (SAM) to acceptor DNA (Sheid et al 1968) and show species and strain specificity (Gold & Hurwitz 1964a). Thus, although a particular DNA methyltransferase is incapable in-vitro of methylating DNA from the same strain, it may be capable of methylating heterologous DNA (Fujimoto et al 1965).

To study the inhibition of a specific prokaryotic DNA methyltransferase by synthetic analogues of *S*-adenosy-L-homocysteine (L-SAH), we have utilized a mutant strain of DP330 *Escherichia coli* J6-2 *dcm*$^+$ that is deficient in DNA cytosine-5-methyltransferase, namely, DP1062 *E. coli* J6-2 *dcm*$^-$. Both the J6-2 *dcm*$^+$ and the J6-2 *dcm*$^-$ strains are derived from *E. coli* strain K-12 (Bachmann 1972). The former organism should therefore carry at least three DNA-base methylation patterns, i.e. the K strain restriction/modification system, which is an adenine methylation system; further adenine methylation determined by the *dam*$^+$ gene, which is involved in the identification of DNA strands in mismatch DNA repair; and cytosine methylation determined by the *dcm*$^+$ gene.This latter activity is absent in the mutant J6-2 *dcm*$^-$ strain. Thus DNA isolated from the mutant cells will be unmethylated at cytosine bases and should be able to function as an in-vitro substrate for DNA cytosine-5-methyltransferase in cell extracts from the parent *dcm*$^+$ strain.

This paper describes an assay system which allows the evaluation of the in-vitro inhibitory properties of L-SAH and a number of structurally related compounds, against *E. coli* DNA cytosine-5-methyltransferase.

MATERIALS AND METHODS

S-Adenosyl-L-homocysteine (L-SAH), *S*-methyl-5′-deoxy-5′-thioadenosine (MTA) and *S*-isobutyl-5′-deoxy-5′-thioadenosine (SIBA) were purchased from Sigma. *S*-Adenosyl-D-homocysteine (D-SAH) and *S*-adenosyl-DL-homocysteine (DL-SAH) were

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prepared from 5'-chloro-5'-deoxyadenosine and D- or DL-homocysteine via the method of Borchardt et al (1976). *S*-Tubercidinyl-DL-homocysteine (STH) was prepared from 5'-chlorotubercidin, as previously reported (Crooks et al 1979) and the product purified by preparative high-pressure liquid chromatography on a Magnum-9 ODS column (50 cm × 9·4 mm) (Whatman) using methanol: water (20 : 80 v/v) as mobile phase. *E. coli* strains J6-2 *dcm*$^+$ and J6-2 *dcm*$^-$ are derivatives of the *E. coli* K-12 strain. Their full designations are: DP330 *E. coli* J6-2 *dcm*$^+$ *pro*$^-$ *his*$^-$ *trp*$^-$ *lac*$^-$, and DP1062 J6-2 *dcm*$^-$ *pro*$^-$ *trp*$^-$ *thy*$^-$ *lac*$^-$ (Pinney & Tribé 1977).

Extraction of dcm$^-$ *DNA*

E. coli strain J6-2 *dcm*$^-$ was grown overnight in 500 ml of nutrient broth (Oxoid No. 2) with shaking. The cells were harvested and resuspended to 10 ml in saline EDTA (0·15 NaCl; 0·1M EDTA, pH 8·0) and added to 10 ml of saline EDTA containing 20 mg of lysozyme (Boehringer) at 37 °C. The suspension was incubated at 37 °C for 15 min with gentle shaking. 1·0 ml of a 2% solution of sodium dodecyl sulphate in water was added and the mixture held at 60 °C for 10 min; 5·0 ml of 4M NaCl was added followed by 25 ml of chloroform–isoamyl alcohol (24 : 1). The mixture was transferred to a 50 ml stoppered measuring cylinder and shaken vigorously to emulsify the system. It was then rocked gently for 20 min at room temperature (20 °C). The layers were separated by centrifugation at 4000 rev min^{-1} for 10 min and the top layer (aqueous phase) was transferred to a 100 ml beaker previously cooled on ice. 2 vols of absolute ethanol, previously cooled to −20 °C, were layered gently onto this solution taking care not to mix the layers. Nucleic acid precipitated at the interface and was wound onto a glass rod. Excess ethanol was squeezed from the nucleic acid, which was then dissolved in 3·75 ml of saline sodium citrate (SSC) (0·15 M NaCl; 0·015 M sodium citrate). A solution of bovine pancreas ribonuclease-A (Sigma) at 250 μg ml^{-1} in water was heated at 90 °C for 15 min, and 0·75 ml added to the SSC solution of nucleic acid. After 15 min incubation at 37 °C the DNA was reprecipitated by layering on isopropanol (1·5 vol) and wound onto a glass rod as before. It was then redissolved in 100 mM Tris buffer, pH 8·0 and its concentration determined by spectrometry at 260 nM using a calibration curve prepared from known concentrations of calf thymus DNA in 100 mM Tris buffer pH 8·0. The volume of solution was finally adjusted to give a DNA concentration of 2 mg ml^{-1}, which was stored at −20 °C.

Preparation of crude cell extract from E. coli *strain J6-2* dcm$^+$

E. coli strain J6-2 *dcm*$^+$ was grown overnight without shaking at 37 °C in 200 ml of nutrient broth. The culture was then shaken for 2 h at 37 °C and the cells harvested by centrifugation at 4 °C. Cells were resuspended in 2·4 ml of ice-cold 100 mM Tris buffer pH 8·0 containing 30 mM NaCl and 10 mM mercaptoethanol. This suspension was added to 0·6 ml of glycerol in a 20 ml glass pot and disrupted by ultrasonic disintegration using an MSE Ultrasonic Disintegrator model 150 W Mk 2. The probe was pre-cooled in ice, the glass pot containing the cell suspension kept on ice during disruption, and the peak to peak amplitude of vibration was 6 μm. Two bursts of 30 s duration separated by a 30 s cooling period were used under these conditions to obtain a disrupted cell suspension. This was centrifuged for 30 min at 10 000 *g* at 4 °C and the supernatant (sonicate) stored on ice for immediate use.

Assay of DNA cytosine-5-methyltransferase

The reaction mixture consisted of 200 μl of cell sonicate containing 5·1 mg ml^{-1} protein, 100 μl of *dcm*$^-$ DNA (2 mg ml^{-1}) and 15 μl of [methyl-^{3}H]*S*-adenosyl-L-methionine (SAM) 500 mCi mmol^{-1} (Amersham International) diluted in water from 1 mM to a suitable concentration. The assay was conducted at 37 °C and initiated by the addition of the sonicate to the other two components. 50 μl samples were taken after a 4 min incubation period and deposited onto 2 cm^2 paper strips (Whatman chromatography paper 3MM) which were then immersed in ice-cold 5% aqueous trichloroacetic acid solution (TCA). The samples were given two washes in 5% TCA, each of at least 30 min duration, before final washing, for 5 min each in successively, ethanol, ethanol–ether (1 : 1) and ether. Once dry, papers were placed separately in 5 ml of scintillation fluid [1000 ml toluene: 500 ml absolute ethanol: 63 ml scintafluor (2·5 g POPOP and 100 g PPO, both by Fisons, in 1000 ml toluene)] and radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Initial velocities of reactions performed in the presence and absence of L-SAH and its analogues were obtained using the same freshly prepared cell extract described above. Inhibition constants (K_i's) obtained from these experiments are shown in Table 1. Lineweaver-Burk plots for L-SAH and STH are shown in Figs 2 and 3, respectively.

RESULTS AND DISCUSSION

Fig. 1 shows the relative DNA methylation activity in a cell extract from *E. coli* strain J6-2 *dcm*$^+$, which contains DNA cytosine-5-methyltransferase, assayed in the absence of exogeneous DNA, in the presence of methylated DNA isolated from *E. coli* strain J6-2 *dcm*$^+$, and in the presence of unmethylated DNA (i.e. unmethylated at cytosine residues) isolated from *E. coli* strain J6-2 *dcm*$^-$, using [methyl-^{3}H]SAM as the methyl donor. Incorporation of radioactivity into acid-insoluble material was plotted against time of sampling. A low level of DNA methyltransferase activity was detectable in the cell extract in the absence of exogeneous DNA, and this activity was increased slightly on the addition of fully methylated DNA isolated from *E. coli* strain J6-2 *dcm*$^+$, to the cell extract. However, these activities were far lower than those obtained when unmethylated DNA isolated from *E. coli* strain J6-2 *dcm*$^-$ was provided as substrate. The data in Fig. 1 indicate that the incorporation of [^{3}H]methyl groups into DNA is not linear with respect to time except in the earlier part (0–5 min) of the time curve. This non-linearity is probably due to the accumulation of SAH, a product and feed-back inhibitor of the enzymic reaction, and has been observed in other methyltransferase systems. Thus, kinetic experiments were therefore performed at time points during the linear portion of the reaction. These results indicate that the above system should provide the basis of a methodology for studying the methylation of cytosine residues in *E. coli* DNA.

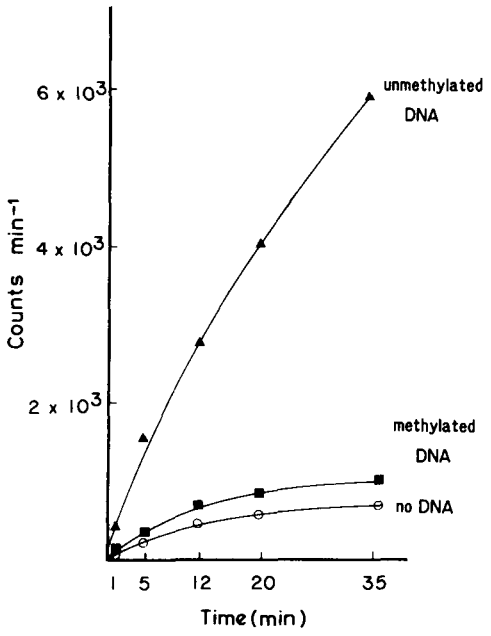


FIG. 1. DNA Methylation in an *E. coli dcm*$^+$ cell extract in the presence of: ○—no added DNA; ■—added DNA isolated from *E. coli dcm*$^+$; and ▲—added DNA isolated from *E. coli dcm*$^-$. The concentration of [methyl-^{3}H]SAM was 20 μM in each experiment.

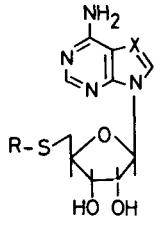
Initial velocity studies were carried out using the J6-2 *dcm*$^+$ cell extract and unmethylated J6-2 *dcm*$^-$ DNA as substrate, and varying concentrations of [methyl-^{3}H]SAM. An apparent K_m value of 15 μM was obtained for SAM in this assay system, which agrees closely with the value reported by Razin et al (1975), for the methylation of DNA using a similar bacterial extract.

Table 1 summarizes the results obtained from the evaluation of L-SAH and five related compounds as inhibitors of *E. coli* DNA cytosine-5-methyltransferase. L-SAH, STH and SIBA were all shown to competitively inhibit cytosine methylation in the above assay system, although SIBA was a much

Table 1. Inhibition constants for L-SAH and related compounds towards *E. coli* DNA cytosine-5-methyltransferase.

Compound	K_i[a] (± s.d. μM)
L-SAH	14·2 ± 2·2
D-SAH	212 ± 55
DL-SAH	63 ± 5·2
SIBA	289 ± 65
MTA	357 ± 71
STH	17·6 ± 2·5

[a] K_i values were determined from double reciprocal plots in which the *S*-adenosyl-L-methionine concentration was varied in the presence of three inhibitor concentrations (see Materials and Methods for details).



Compound	X	R
SAH	N	$-CH_2CH_2CH(NH_2)CO_2H$
SIBA	N	$-CH_2CH(CH_3)_2$
MTA	N	$-CH_3$
STH	CH	$-CH_2CH_2-CH(NH_2)CO_2H$

weaker inhibitor than either L-SAH or STH. MTA was shown to be a poor inhibitor of DNA cytosine methylation. L-SAH has been shown, by several workers, to be a potent competitive inhibitor of bacterial DNA methylation (Gold & Hurwitz 1964b), although no reports are available on its inhibitory activity against individual DNA methyltransferases. The K_i value of 14·2 μM obtained for L-SAH against *E. coli* DNA cytosine-5-methyltransferase indicated that this compound is a potent feed-back inhibitor in the above methyltransferase system (see Fig. 2). This is a surprising finding, considering that SAH is susceptible to metabolic deactivation by prokaryotic SAH nucleosidase, the enzyme that catalyses the cleavage of the glycosyl linkage of SAH, to yield adenine and *S*-ribosylhomocysteine (Duerre 1962).

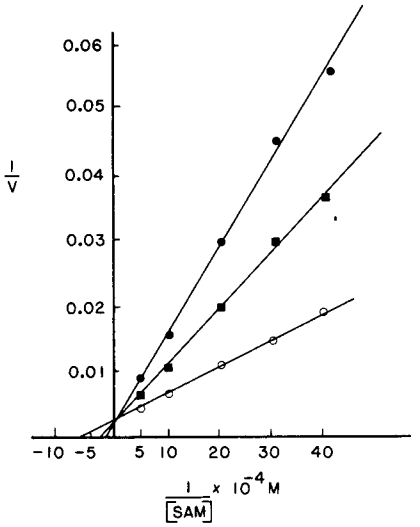


FIG. 2. Lineweaver-Burk plot showing inhibition of *E. coli dcm*+ DNA cytosine methyltransferase by *S*-adenosyl-L-homocysteine at inhibitor concentrations of: 0 (○), 14 (■) and 28 (●) μM. Assay conditions are outlined in the Materials and Methods section. SAM concentration was 2·5–20 μM. *E. coli dcm*− DNA concentration = 1·3 mg ml^{-1}. Vel = d min^{-1} values incorporated mg^{-1} of protein min^{-1}.

In addition, the K_i values obtained for L-SAH, D-SAH, and DL-SAH indicate that a high degree of stereospecificity is shown by the DNA cytosine-5-methyltransferase enzyme towards the L-isomer of SAH, the K_i for this isomer being about a fifteenth the value of that obtained for the D-isomer (see Table 1). The inhibition exhibited by STH, (see Fig. 3), the 7-deaza analogue of SAH, is interesting, and represents the first report of the inhibition of a DNA methyltransferase by this compound. STH has previously been shown to inhibit a wide range of SAM-dependent methyltransferases, both in-vivo and in-vitro, including a number of RNA methyltransferases (Coward & Crooks 1979). It is a metabolically stable methyltransferase inhibitor (Crooks et al 1979), and has comparable inhibitory potencies to L-SAH against many methyltransferase enzymes. The weak inhibitory activity exhibited by SIBA is not unexpected, since this compound has very weak inhibitory activity against isolated SAM-dependent methyltransferases in-vitro (Coward & Crooks 1979), even though it is an effective in-vivo inhibitor of a number of methyltransferases. However, this in-vivo activity may be due to an indirect effect, arising from its inhibitory effect on either SAH hydrolase, the enzyme that catalyses the degradation of SAH to adenosine and homocysteine, or MTA phosphorylase. The poor inhibitory activities of D-SAH, SIBA and MTA suggest that 5′-deoxy-5′-thioadenosine analogues with an intact amino-acid chain bearing the correct chirality may be a structural requirement for potent inhibition of DNA methyltransferase enzymes.

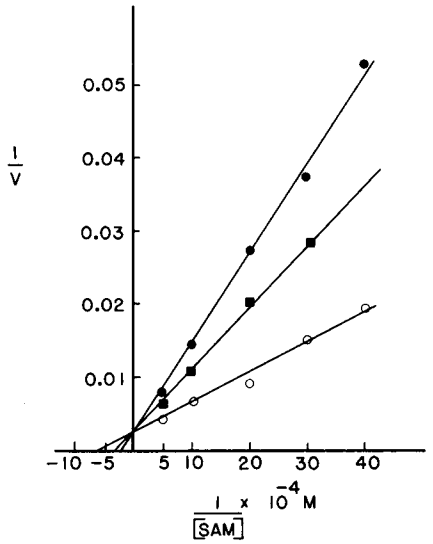


FIG. 3. Lineweaver-Burk plot showing inhibition of *E. coli dcm*+ DNA cytosine methyltransferase by *S*-tubercidinyl-homocysteine at inhibitor concentrations of: 0 (○), 14 (■) and 28 (●) μM. Assay conditions are outlined in the Materials and Methods section. SAM concentration = 2·5–20 μM. *E. coli dcm*− DNA concentration = 1·3 mg ml^{-1}. Vel = d min^{-1} values incorporated mg^{-1} of protein min^{-1}.

CONCLUSIONS

Most prokaryotic DNA methylases are part of restriction-modification systems and protect the host

DNA against degradation by indigenous restriction endonucleases (Arber & Linn 1969; Boyer 1971). Specificity towards restriction endonuclease activity is dependent upon the presence or absence of methyl groups within particular nucleotide sequences. Usually, absence of methylation in these 'recognition sites' results in cleavage of DNA either within the site or elsewhere on the biopolymer. The biological role of *E. coli* DNA cytosine methylase has not been fully elucidated, but *E. coli* mutants devoid of the cytosine methylase activity (i.e. *dcm*$^-$) are susceptible to restriction by the Eco RII endonuclease determined by certain bacterial plasmids (Pinney & Tribé 1977). In addition, these mutants no longer exhibit the two specific 2-aminopurine mutational 'hot-spots' in the *lac I* gene (Coulondre et al 1978). These have been attributed to enzymatic deamination of 5-methylcytosine residues to thymidine residues, a transformation that could give rise to spontaneous mutations (Grippo et al 1970).

The use of bacterial mutants devoid of a single DNA methylase offers a unique opportunity for evaluating inhibitors of specific DNA methylases since it permits the application of an homologous assay system i.e. one which utilizes methylase and substrate from the same organism. Although the well characterized and commercially available Hpa II methylase (New England Biolabs) has been used in DNA methylation studies, this enzyme utilizes a heterologous source of DNA (e.g. *E. coli* or lambda), and it is not known whether the substrate characteristics of heterologous DNA are the same as those of homologous DNA. The isolated hypomethylated mutant DNA from *E. coli dcm*$^-$ is an excellent substrate for the DNA cytosine-5-methyltransferase present in the crude cell extract from the wild type *dcm*$^+$ cells. Our finding that STH is an inhibitor of *E. coli* DNA cytosine-5-methyltransferase, indicates that this metabolically stable inhibitor may be a useful tool in elucidating the role of DNA cytosine methylation in prokaryotic cell function, and in determining the potential of inhibitors of this type as antibacterial agents.

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