ENZYMATIC DNA MODIFICATION IN VITRO: CYTOSINE METHYLATION IN HETEROLOGOUS DNAs BY DIFFERENT DNA METHYLASE FRACTIONS FROM *E. COLI*

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1. Introduction

The biological function of the majority of methylated bases in DNAs is still unknown [1,2].

Recently Roy and Smith purified and characterized four DNA-adenine methylases from *Hemophilus influenzae* Rd [3]. Evidently, the bacterial cell also contains several DNA-cytosine methylases which methylate cytosine in several nucleotide sequences.

The present study in an attempt to elucidate the possible multiplicity of DNA-cytosine methylases in bacteria. To this end, a study was made of the pattern of cytosine methylation in heterologous DNAs methylated by a cell-free extract and different fractions of DNA-cytosine methylases from *Escherichia coli* MRE 600 obtained in the course of its purification. The present work describes the procedure used to separate two DNA-cytosine methylase activities. The specificity of cytosine methylase activities was studied by analysis the 5meCyt* (formed in vitro) distribution in pyrimidine isopliths of heterologous DNAs. The data obtained show the existence of at least two different nucleotide sequences containing 5meCyt and some cytosine methylase fractions from *E. coli* MRE 600 producing modifications of cytosine in these sequences.

* *Abbreviations:* Cyt = cytosine; 5meCyt = 5-methylcytosine; Pur = purine; Pyr = pyrimidine.

2. Materials and methods

The crude extract (165 000 *g* supernatant) from *E. coli* MRE 600 cells (150 g) was prepared as described previously [4]. All subsequent centrifugations were carried out at 40 000 *g* for 10 min. All operations were conducted at 0–5°C. The buffers contained 0.01 M 2-mercaptoethanol.

To 350 ml of 165 000 *g* supernatant were added 7 ml of 10% streptomycin sulfate. The suspension was centrifugated and the supernatant collected (fraction 1). To 355 ml of fraction 1 were added 79 g $(NH_4)_2SO_4$ to obtain 40% saturation. The supernatant was collected as above and brought to 60% saturation with 123 mg/ml $(NH_4)_2SO_4$. The resulting precipitate was redissolved in 80 ml of 0.002 M potassium phosphate buffer, pH 7.7 (0.002 M phosphate buffer) and dialysed extensively against the same buffer (fraction 2). Fraction 2 was diluted to 400 ml with 0.002 M phosphate buffer and applied at 50 ml/hr to a DEAE-Sephadex A-50 column (90 cm × 2.9 cm) that had been equilibrated with 0.002 M phosphate buffer. The column was washed with 500 ml of the same buffer, 500 ml of 0.02 M phosphate buffer and eluted at 50 ml/hr with a linear 2-litre gradient of 0.02–0.42 M phosphate buffer. The DNA-cytosine methylase activity peak was found in 360 ml of eluate with a mean phosphate concentration of 0.12 M. The fraction was concentrated by adding 440 mg/ml $(NH_4)_2SO_4$. The precipitate was dissolved in 30 ml of 0.02 M phosphate buffer and dialysed against the same buffer (fraction 3). Fraction

3 was applied at 30 ml/hr to a column (15 cm × 1.5 cm) of Whatman P11 phosphocellulose that had been equilibrated with 0.02 M phosphate buffer. The column was washed with 80 ml of 0.02 M phosphate buffer and eluted at 30 ml/hr with a linear 500 ml gradient of 0.02–0.52 M phosphate buffer. Two DNA-cytosine methylase activity peaks (fractions I and II) were found with a mean phosphate concentration of 0.18 M and 0.23 M (volumes of 45 ml and 40 ml respectively). These two fractions were concentrated by ultrafiltration in a Diaflo Amicon pressure cell with a PM 10 membrane and the solvent was changed to 0.02 M phosphate buffer. The volume of the two final fractions was 5 ml. An equal volume of cold glycerol was added to each fraction and they were stored at −20°C.

Heterologous DNAs were methylated by different fractions of DNA-cytosine methylases up to complete saturation with the methyl groups. The incubation mixture (1 ml) contained: 40 μmoles of Tris buffer, pH 8.0; 2 μmoles of $MgCl_2$; 4 μmoles of 2-mercaptoethanol; 0.01 μmoles of [methyl-^{14}C] *S*-adenosylmethionine (Amersham; specific activity 55 mCi/mmole); 0.3 μmoles of DNA and DNA-methylase (up to 50 units). (One unit of DNA-methylase activity is defined as that amount of enzyme which, under the standard assay conditions, incorporates 1 pmole of methyl groups into DNA in 1 hr). The mixture was incubated at 37°C for 40 min with the cell-free extract, 90 min with fraction 3 and 120 min with phosphocellulose fractions I and II. The reaction was stopped and DNA isolated as described previously [5]. The DNAs obtained were degraded to pyrimidine sequences by Burton's method [6]. The released isopliths of general formula of Pyr_nP_{n+1} were separated on DEAE-Sephadex A-25 according to chain length and the content of 5meCyt formed in vitro in each peak was determined as previously [5]. Dipyrimidine isopliths were separated according to the base compositions [7].

3. Results and discussion

3.1. *The discrepancy between homologous and heterologous DNA methylation*

The data on the distribution of 5meCyt formed in vitro in pyrimidine isopliths of DNAs of *E. coli* B, *Pseudomonas fluorescens* and calf thymus methylated by cell-free extract and some fractions of *E. coli* MRE 600 methylase are listed in table 1. This table also

Table 1
Occurrence frequency of 5meCyt in pyrimidine isopliths of heterologous DNAs methylated by different DNA methylase fractions of *E. coli* MRE 600

DNA methylase source	DNA source	5meCyt of total 5meCyt (%) — Isopliths I	II	III	IV	V	>V
Cell-free extract (165 000 *g*)	*E. coli* B	1.55	87.26	6.31	2.11	1.33	1.44
	Ps. fluorescens	1.18	84.30	9.15	2.78	1.12	1.47
	Calf thymus	0.87	26.22	38.29	16.93	6.04	11.65
DEAE-Sephadex	*E. coli* B	0.64	93.79	2.97	1.03	0.55	1.02
	Ps. fluorescens	1.58	87.82	5.83	1.92	0.78	2.07
	Calf thymus	5.25	51.21	16.01	7.80	5.53	14.20
Phosphocellulose Fraction I	*E. coli* B	1.77	96.08	1.09	0.36	0.24	0.46
	Ps. fluorescens	6.91	91.61	1.07	0.05	0.24	0.12
	Calf thymus	31.58	61.37	1.81	0.89	2.34	2.01
Phosphocellulose Fraction II	*Ps. fluorescens*	7.69	91.16	0.49	0.05	0.57	0.04
	Calf thymus	11.06	80.45	4.27	0.49	0.35	3.38
–	*E. coli* MRE 600 DNA methylated in vivo	2.68	44.60	27.54	12.48	5.75	6.95

contains data on the frequency with which 5meCyt occurs in pyrimidine isopliths of *E. coli* MRE 600 DNA methylated in vivo [8]. When comparing all DNAs methylated by the cell-free *E. coli* MRE 600 extract, attention should be paid to the similarity of all DNAs in having negligible cytosine methylation in a solitary position, i.e. in the . . . Pur–Cyt–Pur . . . sequence. *E. coli* B and *Ps. fluorescens* DNAs reveal significant similarity in their pattern of 5meCyt distribution. About 90% of the total 5meCyt was found in pyrimidine dinucleotide clusters in two heterologous bacterial DNAs. Calf thymus DNA differs from bacterial DNAs in its pattern of 5meCyt distribution in oligopyrimidine clusters. *E. coli* MRE 600 DNA, methylated in vivo, differs from all heterologous DNAs, methylated in vitro, in its 5meCyt distribution in pyrimidine oligonucleotides. Proceeding form the similarity of DNA primary structures in the two *E. coli* strains and the absence of 5meCyt in *E. coli* B DNA [9], the pattern of cytosine methylation in *E. coli* B DNA in heterologous DNA methylase reactions may be assumed to be the same as that one in *E. coli* MRE 600 DNA. However, the observed 5meCyt distribution in *E. coli* B DNA does not bear out this assumption.

Probably, several DNA-cytosine methylases, which methylate cytosine in different nucleotide sequences are present in *E. coli* MRE 600 cells, and the actions of these enzymes, controlled in vivo, are disturbed in vitro due to competing DNA methylase interactions. This may explain the difference between the pattern of the 5meCyt distribution in oligopyrimidine sequences of all heterologous DNA, in particular *E. coli* B DNA, and the pattern of homologous methylation of *E. coli* MRE 600 DNA. The data on the analysis of heterologous DNAs methylated by purified fractions of DNA-cytosine methylase are in agreement with this assumption.

3.2. *The existence of some DNA-cytosine methylases in E. coli MRE 600*

The *E. coli* MRE 600 DNA methylase system is enzymatically active in adenine and cytosine methylation [8]. The present work describes the procedure used to distinguish certain DNA-cytosine methylase activities from DNA-adenine methylase. Table 2 summarizes the results for DNA-cytosine methylase purification. The phosphocellulose step of purification made it possible to single out two cytosine methylase activities.

It is important to note that calf thymus DNA is a good substrate to show up the specificity of different DNA-cytosine methylase fractions. As a result of this DNA methylation by cytosine methylase fractions the maximum 5meCyt content shifts to pyrimidine dinucleotides in . . . Pur–Cyt–Cyt–Pur . . . sequence. When the calf thymus DNA is methylated by phosphocellulose cytosine methylase fractions I and II, the solitary cytosine methylation increases significantly (up to 31.59% and 11.06% respectively). The calf thymus DNA preliminarity methylated in vitro by DEAE-Sephadex fraction was incubated under standard conditions (see Materials and methods) with phosphocellulose fraction I in the presence of unlabelled *S*-adenosylmethionine.

Table 2
Purification of DNA-cytosine methylase activity of *E. coli* MRE 600

Purification stage	Protein (mg)	Activity (units)		Specific Activity (units/mg)	
		CMA	AMA	CMA	AMA
Supernatant 165 000 *g*	2773	54 628	28 562	19.7	10.3
Streptomycin sulfate	2765	66 360	34 562	24	12.5
$(NH_4)_2SO_4$	1514	56 775	34 065	37.5	22.5
DEAE-Sephadex A-50	72.03	6 288	187	87.3	2.6
Phosphocellulose P_{11}					
Fraction I	0.96	3 301	10	3438.5	10.3
Fraction II	0.89	2 318	7	2605	7.8

Ps. fluorescens DNA was used as substrate
CMA - cytosine methylase activity
AMA - adenine methylase activity

No visible increase of 5meCyt was observed in pyrimidine mononucleotides. This experiment shows that the high 5meCyt content in a solitary position in calf thymus DNA methylated by phosphocellulose cytosine methylase fractions does not result from nuclease action.

The same characteristic pecularity of corresponding fractions of DNA-cytosine methylases (though not so pronounced) is observed also with methylated bacterial DNAs.

The above data show that each DNA-cytosine methylase fraction differs in its specific capacity for methylating heterologous DNAs. The methylated pyrimidine oligonucleotides may be considered as fragments of different nucleotide sequences which are recognized by specific DNA-cytosine methylases. Therefore, the DNA-cytosine methylases which methylate the specific nucleotide sequences, from which the hydrolysis according to Burton releases methylated pyrimidine dinucleotide and mononucleotide, are enriched in the course of purification. These results demonstrate the existence of at least two nucleotide sequences containing 5meCyt (. . . Pur–Cyt–Pur . . . and . . . Pur–Cyt–Cyt–Pur . . .) and some cytosine methylase fractions from *E. coli* MRE 600 producing modification of cytosine in these sequences.

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