Stimulation of de novo methylation following limited proteolysis of mouse ascites DNA methylase

R.L.P. Adams, R.H. Burdon, K. McKinnon and A. Rinaldi

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

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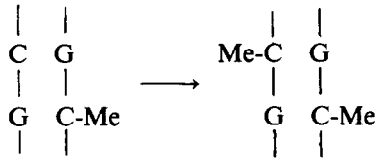
The ability of mouse Krebs II ascites cell DNA methylase to add methyl groups to native, unmethylated DNA (de novo activity) is stimulated by limited proteolysis. The affinity of the enzyme for DNA is not altered by this treatment but the rate of reaction is increased so that 40% or more of methylatable sites are methylated within 4.5 h. The activation is associated with a decrease in size of the enzyme to 6.2 S.

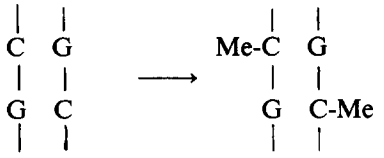
De novo methylation *Proteolysis* *DNA* *Methylase*

1. INTRODUCTION

Methyl groups are added to certain cytosines in CG dinucleotides in DNA shortly after DNA synthesis. The signal that determines which cytosines are to be methylated is probably the presence of a methylcytosine in the parental DNA and this leads to the maintenance of the pattern of methylation of the parental cell in the genes of the daughter cells [1–4].



There is a strong negative correlation between the methylation of a gene and its expression [5,6] though whether demethylation results in gene expression or the reverse is by no means clear. Whichever is the case, there are situations in which methyl groups are lost from the DNA – probably by replication in the absence of methylation – and to compensate for this there are times when methyl groups must be added to completely unmethylated regions of DNA – so called de novo methylation.



Little de novo methylation occurs on foreign DNA introduced into somatic cells in culture. However, the existing pattern of methylation of the input DNA is maintained with high fidelity for 25 generations [7]. On the other hand, similar experiments with embryonal carcinoma cells (a model of undifferentiated preimplantation embryo cells) or cells of very early embryos indicate that de novo methylation of input foreign DNA can occur in such undifferentiated cells [8–10].

We have been studying this problem at the level of the enzymic capabilities of the DNA methyl transferase which has been partially purified from mouse Krebs II ascites tumour cells. This enzyme exhibits activity on unmethylated and hemimethylated substrates; i.e., it has both de novo and maintenance activities though the activity is greatest on hemimethylated substrates [11,12]. We report here that the de novo methylase activity can be stimulated up to 30-fold by limited proteolysis and that the treatment probably brings

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about a decrease in the size of the enzyme. The value of its sedimentation coefficient is reduced from over 7 S to 6.2 S.

2. MATERIALS AND METHODS

DNA methylase was purified from nuclei of Krebs II ascites tumour cells by modifications of methods in [11,13]. Briefly an 0.2 M NaCl nuclear extract (fraction 1) was absorbed onto phosphocellulose and released with 0.5 M NaCl (fraction 2). This fraction was precipitated with ammonium sulphate at 50% saturation (fraction 3) and applied to a column of Ultrogel AcA 34. The enzyme (fraction 4) elutes with a kDa of 1.5 and is adsorbed onto heparin–Sepharose from which it is released with 0.7 M NaCl (fraction 5). Details of the purification will be published later.

The enzyme was assayed as in (Adams and Burdon, 1983) using *Escherichia coli* of *M. luteus* DNA (Sigma) or DNA purified from mosquito or mouse L929 cells [13]. Hemimethylated DNA was isolated from L929 cells maintained for one S phase in azadeoxycytidine (10^{-6} M) [14,15].

Glycerol gradients (15–30% glycerol containing 0.2 M NaCl) were prepared and run as in [11] with markers of yeast alcohol dehydrogenase (7.0 S) and haemoglobin (4.1 S).

3. RESULTS

3.1. *Stimulation of de novo methylase activity by limited proteolysis*

As shown in fig.1 the activity of mouse Krebs II ascites cell DNA methylase is increased following brief treatment with trypsin or chymotrypsin. The optimal protease concentration is less than 20 μg/ml but perhaps more importantly the ratio of enzyme protein to protease for maximal stimulation is between 100 and 300 to 1.

The degree of stimulation is variable but is seldom less than 3-fold and can be as much as 30-fold and this variability may be a result of prior unintentional protease activity which cannot be excluded despite the addition of phenyl methyl sulphonyl fluoride (PMSF) to all enzyme preparations. The stimulation is restricted largely to activity on a native unmethylated DNA substrate and is much less obvious with a denatured or a hemimethylated DNA substrate (table 1).

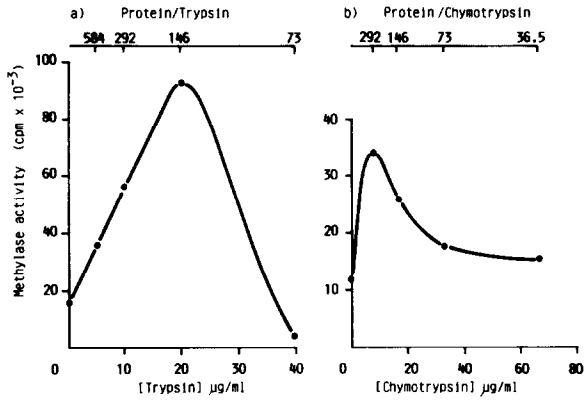

Fig.1. Effect of proteases on de novo methylase activity. (a) 73 μg methylase fraction 4 was incubated with trypsin for 10 min at 37°C and the action stopped with a 5-fold excess of trypsin inhibitor. (b) 36.5 μg methylase was incubated with chymotrypsin and the action stopped with PMSF (1 mg/ml). DNA methylase activity was measured using native *E. coli* DNA.

3.2. *Characteristics of the activated enzyme*

The stimulation of methylase activity is seen whether DNA is in excess or whether DNA is present in limiting amounts (fig.2). In fig.3a it can be seen that the reaction continues for several hours with supercoiled ϕX174 RF DNA leading to methylation at 4.5 h of over 40% of the cytosines in CG dinucleotides. (Similar results have been obtained with DNA from phage lambda and complete methylation of SV40 DNA has been obtained following 3 h incubation.) By 4.5 h time the ϕX174 RF DNA shows considerable resistance to digestion with the restriction enzyme *Hpa*II indicating methylation of the internal cytosine in the CCGG recognition site (fig.3b). However, no material resistant to *Msp*I digestion is visible on ethidium-stained gels or the corresponding fluorogram indicating that no methyl groups are added to the 5′ cytosine in the CCGG recognition site of *Msp*I.

Two things should be noted. Firstly, as shown in control incubations run in parallel with normal unmethylated ϕX174 RF DNA, the *Hpa*II digestion goes to completion (not shown). Five fragments are produced of 2745, 1690, 374, 348 and 218 base pairs. Thus the result in fig.3b slot H is not caused by insufficient enzyme but by blockage of enzyme action by methylation.

Table 1

Effect of trypsin treatment on DNA methylase activity measured with different substrates

Enzyme protein/trypsin	Experiment A		Experiment B	
	Control	251	Control	191
Methylase activity (cpm)				
Native DNA	640	7300	1180	14300
Denatured DNA	2710	3130	–	–
Hemimethylated DNA	–	–	4470	10100
Stimulation				
Native DNA	11.4		12.1	
Denatured DNA	1.2		–	
Hemimethylated DNA	–		2.3	

25–30 μg methylase (fraction 4 or 5) was incubated with 0.1 μg trypsin (14 μg/ml) as described for fig.2. Activity was assayed using 1 μg native or denatured DNA from *Aedes* or hemimethylated L929 cell DNA

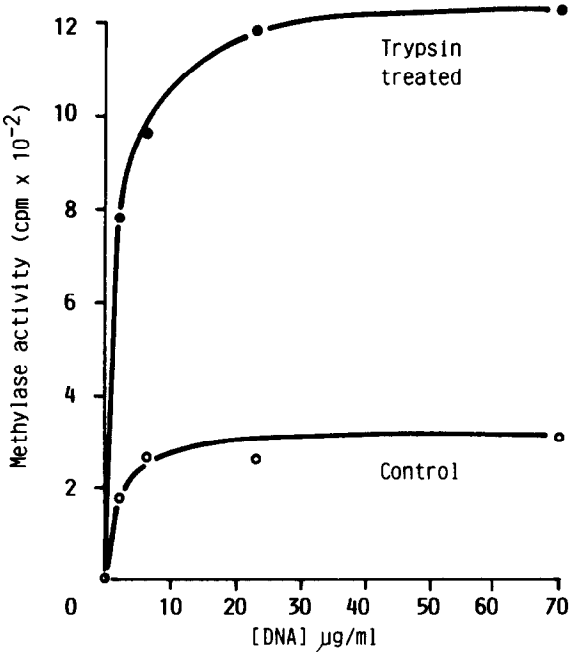

Fig.2. DNA concentration curve for activated methylase. 73 μg methylase fraction 4 was incubated for 10 min at 37°C with 0.5 μg trypsin (20 μg/ml) and the action stopped with a 5-fold excess of trypsin inhibitor. Methylase activity was assayed using 4.8 μg enzyme protein and native *M. luteus* DNA.

Secondly, as can be seen from the fluorograph, whilst some methylation coincides with the form II DNA, implying that all 5 *Hpa*II sites of ϕX174 RF DNA can be methylated, the predominant methylated band corresponds to full length linear ϕX174 DNA. Thus one *Hpa*II site appears relatively resistant to methylation.

Despite these differences the sensitivity of the trypsin-treated DNA methylase to increasing NaCl

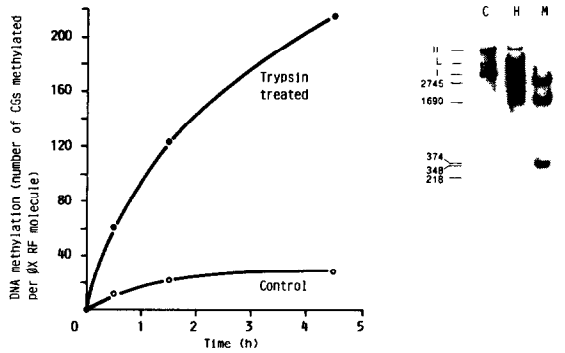

Fig.3. Time course of methylation of viral DNA. 73 μg enzyme was activated as described for fig.2 and used to methylate 2 μg ϕX174 RF DNA. (a) Time course of methylation. (b) Fluorogram of DNA methylated for 4.5 h, treated with: M, *Msp*I; H, *Hpa*II; C, unrestricted, and separated by electrophoresis on 1.5% agarose.

concentrations in the assay with native DNA closely parallels that previously encountered with the untreated enzyme [13] (not shown). Moreover, the effect of NaCl (200 mM) added once the methylation reaction is underway, is to block further methylation immediately. In this respect the reaction is similar with trypsin-treated and untreated enzyme and argues against anything more than a very limited procession of either form of enzyme along the duplex DNA.

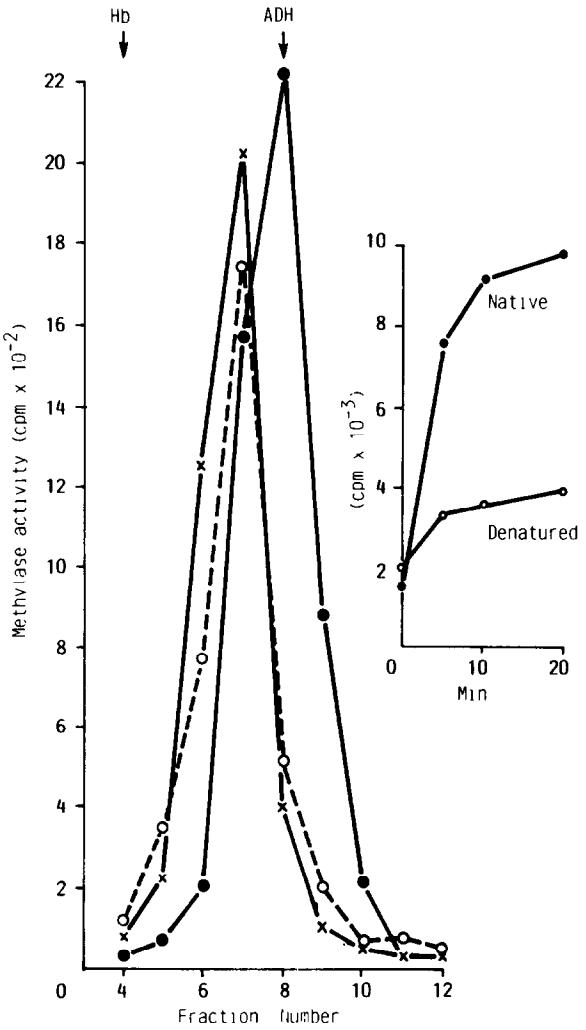

Fig.4. Glycerol gradient analysis of trypsin-treated methylase. DNA methylase (2.16 mg fraction 3) was treated with trypsin (6 μg) for different times. The inset shows the time course of activation using a native or denatured *E. coli* DNA substrate (23 μg enzyme protein per assay). Samples (422 μg enzyme protein) were run on 15–30% glycerol gradients containing 0.2 M NaCl (4 ml gradients were centrifuged at 45 000 rpm in a Beckman SW56 rotor for 16 h). Internal markers of yeast alcohol dehydrogenase (ADH) and haemoglobin (Hb) were used. The gradients were collected by upward displacement and samples (40 μl) were assayed using denatured *E. coli* DNA as substrate. Time of trypsin treatment: (●) zero; (×) 5 min; (○) 20 min.

Both untreated and trypsin-treated enzyme have a similarly high affinity for native *M. luteus* DNA, both showing a K_m of about 2 μg/ml (fig.2). The increase in maximum velocity observed may thus reflect an increased rate of transfer of methyl groups from *S*-adenosyl methionine, or an enhanced ability to recognise and act on an unmethylated site in native DNA.

3.3. *The effect of trypsin on the size of DNA methylase*

We have previously reported a size of 8.3 S for mouse ascites DNA methylase as measured on glycerol gradients containing 0.2 M NaCl [11]. This would correspond to a M_r-value of about 180000 for a globular protein. Fig.4 shows the results of glycerol gradient fractionation of mouse ascites DNA methylase after increasing times of trypsin action. Initially the enzyme sediments faster or in the same position as yeast alcohol dehydrogenase (7.0 S) but after limited treatment with trypsin (enzyme:protease ratio = 500) the enzyme sediments more slowly than alcohol dehydrogenase and has a peak at 6.2 S. These changes are associated with a steadily increasing activity with a native DNA substrate relative to a denatured DNA substrate (see insert fig.4).

4. DISCUSSION

The activation of DNA methylase activity following limited proteolysis raises questions regarding the mode of action of the enzyme as isolated normally from Krebs II mouse ascites tumour cells. It is normally a high-M_r protein and is probably typical of the DNA methylase activity present in intact somatic cells where, as judged from its preference for hemimethylated DNA [12], it probably serves to maintain the cellular pattern of DNA methylation. For this function the enzyme appears to be associated with the nuclear matrix in a complex with other enzymes involved in DNA replication [16,17].

This preference for hemimethylated DNA as substrate suggests the possible recognition of a bipartite structural element in the substrate DNA. Whilst the enzyme has to methylate CG sequences its specificity for such targets may require the recognition of a base-paired methylated CG sequence on the opposite strand. The lower activity of the enzyme with unmethylated double-stranded DNA as substrate may then be due to the presence in the opposite strand of unmethylated rather than methylated CG sequences. This might impair proper catalytic function or even have a suppressive effect. This latter view is not unreasonable in view of the observation that single-stranded DNA is a good substrate for the enzyme. In this situation the absence of any CG sequence base-paired in a complementary location might simply allow the methylase to operate without impediment.

Our observation that mild proteolysis stimulates only the activity detected with unmethylated double-stranded DNA suggests that the enzyme has now become more relaxed with regard to DNA structural requirements. The fact that this change in property is accompanied by a reduction in the sedimentation coefficient is of interest. It is unlikely that trypsin treatment is simply removing a non-specific inhibitor as its effects are only seen when unmethylated native DNA is used as substrate. Moreover, the response to proteolytic digestion is seen at all levels of purification. A possible explanation is that mild proteolysis removes or alters some 'domain' of the enzyme such as to promote an increase in the level of de novo methylation of double-stranded DNA.

From a biological standpoint the occasional switch at various times in development from the normal maintenance mode of DNA methylase might thus be achieved by specific proteolysis. In teratocarcinoma cells or early embryos where there seems to be high de novo methylating activity, this could clearly result from controlled proteolytic maturation in vivo and this possibility is presently under investigation.

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