FEBS Letters 460 (1999) 380–384 FEBS 22816

Characterization of a new variant DNA (cytosine-5)-methyltransferase unable to methylate double stranded DNA isolated from the marine annelid worm *Chaetopterus variopedatus*

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Received 9 September 1999; received in revised form 5 October 1999

Abstract The enzyme S-adenosylmethionine-DNA (cytosine-5)-methyltransferase has been identified, first time for invertebrates, in embryos of the marine polychaete annelid worm *Chaetopterus variopedatus*. The molecule has been isolated from embryos at 15 h of development. It is a single peptide of about 200 kDa molecular weight, cross-reacting with antibodies against sea urchin DNA methyltransferase. The enzymatic properties of the molecule are similar to those of Dnmt1 methyltransferases isolated from other organisms, but with the peculiarity to be unable to make 'de novo' methylation on double stranded DNA.

Key words: DNA methyltransferase; Invertebrate; Annelid polychaete; 5-Methylcytosine; *Chaetopterus variopedatus*

1. Introduction

The enzymatic post-replicative methylation of eukaryotic genomic DNA at position 5 of cytosine of CpG dinucleotides has been shown to participate in a number of biological mechanisms such as those controlling gene expression [1], developmental processes [2], chromatin packaging [3] and genomic imprinting [4]. Recently it has been shown that the DNA of the marine worm *Chaetopterus variopedatus* is methylated [5].

The enzyme responsible for DNA methylation, S-adenosylmethionine-DNA (cytosine-5)-methyltransferase (DNA-MTase) has been isolated as cDNA from vertebrates [6–9] and from the sea urchin [10]. Recently other forms of DNA-MTase have been isolated in mammals [11–13]. All those enzymes perform, in addition to maintenance methylation on hemimethylated DNA, also 'de novo' methylation on single (ss) and double stranded (ds) DNA. No data about this enzyme have yet been provided in invertebrate organisms such as worms. Here we describe for the first time the properties of a DNA-MTase present in a marine annelid. We show that while the molecular weight and the fundamental catalytic properties of the enzyme are similar to those of Dnmt1 type DNA-MTase of vertebrate organisms, it has the unexpected and distinctive difference to lack 'de novo' methylation activity on ds DNA. Since the enzyme cross-reacts with antibodies against sea urchin Dnmt1 it has been possible to study its catalytic properties and to measure its molecular weight on the molecule purified by immunoprecipitation. This has permitted to exclude that the inability to methylate ds DNA is due to factors present in the soluble fraction of the embryo homogenate. As a consequence, this property appears to be a peculiarity that distinguishes this enzyme from all other DNA-MTases.

2. Materials and methods

2.1. Collection of eggs, embryonic culture and enzyme extraction

Annelid worms *C. variopedatus* were collected in the bay of Naples by the Fishery Service of the Zoological Station of Naples. Embryonic cultures were prepared as reported [5]. The enzyme was extracted from embryos at different times of development and the activity was determined following the procedure reported for sea urchin DNA-MTase [14]. The 15 h embryos were chosen as a source of the enzyme. No attempt to purify the molecule in the centrifuged homogenate was made because of its very low content in the embryos, the scarcity of the organism and the period of sexual activity limited to the months of July and August. An ammonium sulphate precipitation step was made between 33 and 90% saturation to concentrate the proteins of the centrifuged homogenate to measure the molecular weight of the native enzyme by gel filtration. To this aim, about 15 mg of proteins of the ammonium sulphate pellet were suspended in 1.5 ml of 6.7 mM DTT, 10% glycerol, 100 mM Tris-HCl pH 7.5, 10 mM EDTA, centrifuged at 14 000 rpm for 30 min and the clear supernatant was loaded directly on a Sephadex G-100 column 1.6×100 cm, equilibrated and eluted at a rate of 0.75 ml/min with the same solution buffer. Protein concentration values in the samples were determined with the BioRad reagent kit.

2.2. Antibody preparation and immunoprecipitation of the enzyme

Polyclonal antibodies against the initial 100 N-terminal amino acid residues of the sea urchin DNA-MTase were prepared by inserting the 300 bp residues of cDNA coding for the N-terminal domain of DNA-MTase from *P. lividus* sea urchin (10) in the expression vector pMAL-c2 (BioLabs, New England) as a fusion protein with maltose binding protein. The fusion protein was expressed in *Escherichia coli* TG1 and purified by affinity chromatography on amylose column, according to the manufacturer's instructions. Three aliquots of 400 μg of fusion protein in 500 μl of isotonic saline solution, mixed with an equal volume of Freund's complete adjuvant, were injected at intervals of 15 days to immunize rabbits. Polyclonal antibodies were isolated from the immune sera by ammonium sulphate fractionation followed by DEAE column chromatography [15]. Four mg of *Staphylococcus aureus* protein A-Sepharose CL-4B beads (Pharmacia) were suspended in 1 ml of 100 mM Tris pH 7.5, 10 mM EDTA, 0.5 mM PMSF, 10 mM DTT. The swollen and equilibrated beads were sedimented and the pellet was mixed with 35 μg of the prepared polyclonal antibodies. 200 μg of proteins of the centrifuged homogenate of *C. variopedatus* embryos were added and the volume was brought to 300 μl with the equilibration buffer. The suspension was kept under gentle stirring for 2 h at 4°C and the beads were then separated with a 20 s centrifugation pulse at 4°C in an Eppendorf microfuge. The immunodepleted supernatant was saved to measure residual enzyme activity and to analyze the protein content by SDS-PAGE. The pellet of sedimented beads was washed two times by centrifugation with 300 μl of the

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PII: S0014-5793(99)01379-4

equilibration buffer and used either directly for enzymatic assays or suspended in SDS buffer to analyze by Western blot the proteins dissociated from the pellet.

2.3. Electrophoretic fractionation and Western blot analysis of the enzyme

Proteins were separated by SDS gel electrophoresis on acrylamide gel as reported [16], with the following modifications: acrylamide concentration was 7.5% and acrylamide-bisacrylamide ratio 19:1 to improve the separation of high molecular weight components. Molecular weight protein markers were from FMC (Rockland, ME, USA). After the electrophoretic separation proteins were electrophoretically transferred to a nitrocellulose membrane (Sartorius). Blots were stained with Ponceau Red to analyze the protein content and to confirm equal protein loadings in the different lanes. The immunoenzymatic reactions were performed using rabbit polyclonal antibodies prepared against the N-terminal 100 amino acid residues of sea urchin DNA-MTase [10] and goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich). The proteins bound to the pellets were analyzed by adding 20 μl of SDS electrophoresis sample buffer [16], incubating in a boiling water bath for 10 min and then loading directly on a SDS gel slab for protein fractionation and Western blot analysis.

2.4. Preparation of various forms of substrate DNAs

Micrococcus luteus DNA (Sigma) was purified and the single stranded form was prepared as already described [14]. Substrate ds DNA in supercoiled, relaxed and linear forms, were prepared from the plasmid clone 3200PS containing H1, H2B and part of H3 histone genes of the repeated cluster of five histone genes identified in *C. variopedatus*, inserted in pBluescript KS− (Stratagene) [17]. The linear form was obtained by digesting the plasmid DNA with the restriction enzyme *Pst*I (Boehringer, Mannheim, Germany) in the conditions specified by the manufacturer. The hemimethylated ds DNA was prepared using as a template the single stranded 3200PS plasmid obtained with M13 K07 helper phage. The second strand was synthesized in the presence of m^5-C dCTP using T3 as a primer, as described [14]. At the end of the reaction the DNA samples were analyzed by gel electrophoresis on 1% agarose in 89 mM Tris-borate, 2 mM EDTA, pH 8.3 (Fig. 2B).

2.5. Enzyme assay

The DNA-MTase activity was determined by measuring the incorporation of ^{3}H from [methyl-^{3}H]adenosylmethionine (SAM) (Amersham, 15 Ci/mmol) into DNA-m^5-C, as described [14]. Each data point is the average of two experiments with values differing less than 10%. Enzyme activity in the immunoprecipitated pellet was determined by suspending each batch sample, prepared as described under immunoprecipitation of the enzyme, in 50 μl of assay buffer and using aliquots of 10 μl for each enzyme test.

2.6. HPLC analysis to identify methylated bases in substrate DNA

The substrate *M. luteus* ss DNA, after methylation for 2 h in two standard assay mixtures using 20 μg of proteins, were combined and purified using Quiaex II resin (Quiagen). The isolated DNA, containing about 15000 dpm of incorporated ^{3}H-methyl groups, was hydrolyzed to free bases by incubating in 70% perchloric acid at 60°C for 24 h. An aliquot of about 6000 dpm of hydrolyzed DNA, corresponding to about 4.5 pmol of ^{3}H-methyl groups, was loaded on a HPLC column and fractionated exactly as described [5]. HPLC analysis of standard m^5-C (Sigma) was performed in the same conditions alone and in mixture with the hydrolyzed sample DNA. The presence of radioactivity in the column eluate, due to methyl group incorporation, was measured on 0.5 ml of each eluted fraction, adding 5 ml of Instagel for liquid scintillation assay (Packard) and counting in a Beckman model LS701 spectrometer.

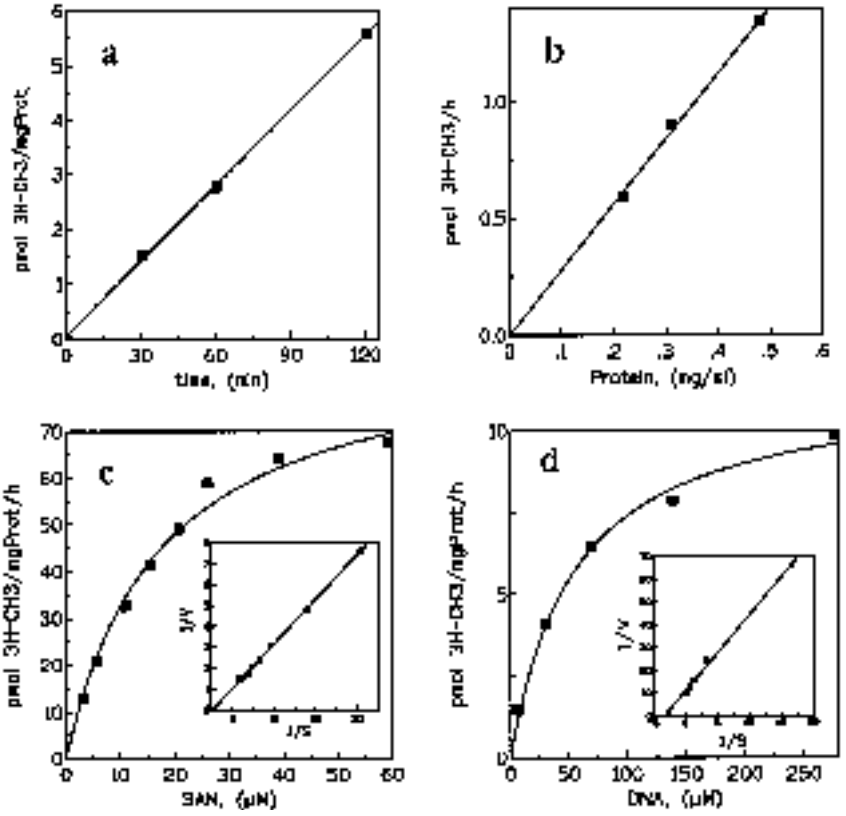

Fig. 1. Enzymatic parameters of *C. variopedatus* DNA-MTase activity using ss *M. luteus* DNA as a substrate. Panel a: Dependence of incorporated methyl groups on incubation time. Panel b: Dependence of DNA-MTase activity on the protein concentration. Panel c: SAM substrate saturation curve at constant 424 μM DNA concentration. Panel d: DNA substrate saturation curve at constant 100 μM SAM. The corresponding Lineweaver-Burk plots of panels c and d are in the corresponding inserts where the axis values are multiplied by 100. Data in a and b are on enzyme in the centrifuged homogenate sample; data in c and d in the active fractions eluted from the Sephadex G-100 column.

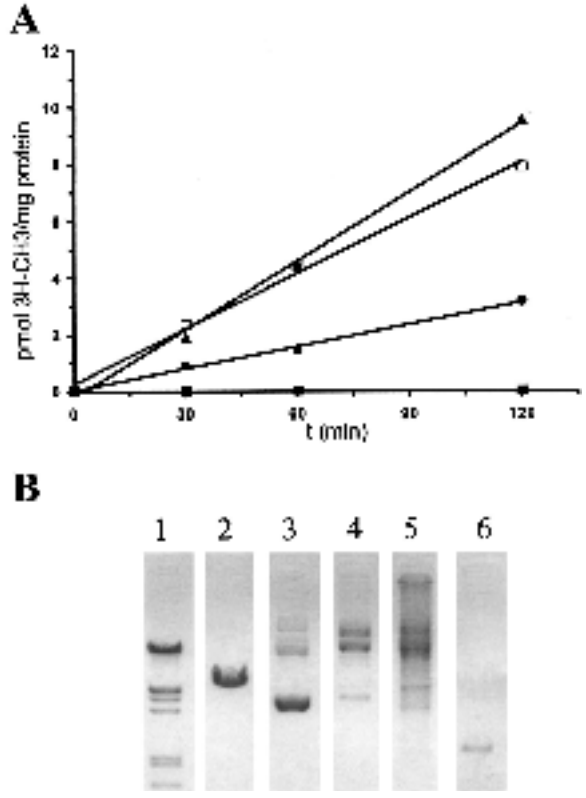

Fig. 2. A: Efficiency of methylation by *C. variopedatus* DNA-MTase of 3200PS plasmid DNA in different topological forms. Enzyme activity in the centrifuged homogenate; 424 μM DNA as substrate. (▲), hemimethylated form, (●), ss form; (■), linear, relaxed and supercoiled ds forms. As references, the time courses of *M. luteus* ss DNA (□) and ds DNA (■) are reported. Note that all ds DNAs, irrespective of their topological forms and origins, are represented by the same line at zero level, indicated by the same symbol. Lines through the data are linear regressions from which the derived relative methylation rates are 1:2.5:6.1 for, respectively, ss plasmid, ss *M. luteus* and hemimethylated ds plasmid DNAs. These values have been calculated considering also the % CpG of those molecules. B: Electrophoretic analysis of substrate DNA. Lane 1, molecular weight DNA markers (Marker III, Boehringer); lanes 2–6, linear, supercoiled, relaxed, hemimethylated and single stranded forms of 3200PS plasmid DNA, respectively.

3. Results and discussion

3.1. Enzyme extraction and analysis of catalytic parameters

The dependence of the enzyme activity on time and protein concentration was determined on the centrifuged homogenate of embryos at the 15 h of development. The substrate saturation curves were analyzed on the enzyme active fractions eluted from the Sephadex G-100 column. The activity value is linearly dependent both on the length of the incubation time (Fig. 1a) and on the concentration of proteins present in the incubation mixture (Fig. 1b). The K_m value for SAM is 21 μM as determined by linear regression analysis of the Lineweaver-Burk plot of a SAM saturation curve (Fig. 1c). This value is substantially higher than 1.4 μM, typical of the mammalian enzymes [18], but very similar to that observed for the sea urchin enzyme, 20 μM [19]. The K_m value for *M. luteus* ss DNA is 31 μM, as determined by regression analysis of the Lineweaver-Burk plot of a DNA saturation curve (Fig. 1d). The ability of the enzyme to methylate various DNA forms was studied on plasmid 3200PS because this permitted to perform experiments on the same DNA molecule in the single stranded form and in various topological states of the ds form including fully hemimethylated ds DNA. The results of the determinations of enzyme activity on the various forms of the same substrate DNA (Fig. 2A) show that methylation occurs at maximal rate on hemimethylated ds DNA although it contains only half of the substrate CpGs of the comparable concentration of the same DNA in the single stranded form, which is methylated at a lower rate. Surprisingly, no methyl group incorporation is observed when the same substrate ds DNA molecule has both strands unmethylated, regardless of its possible topological forms, relaxed, supercoiled and linear. The same negative result is observed when ds *M. luteus* DNA is used.

The enzyme revealed on the Western blot of the immunoprecipitated sample (Fig. 3, lane 3) is enzymatically active and shows the same and identical specificity for the different forms of DNA as the enzyme in the initial centrifuged homogenate. The presence of the enzymatic activity in the immunoprecipitated fraction with antibody preparation against the N-terminal domain of sea urchin DNA-MTase is an indication that also *C. variopedatus* enzyme has the catalytic site in the C-terminal domain [10], like the Dnmt1 enzymes of all other sources [6–9]. The similarities of *C. variopedatus* DNA-MTase with Dnmt1 enzymes extend also to the enzymatic parameters. These well conform to those of Dmnt1 enzymes from *P. lividus* sea urchin [14], mouse in vitro cultivated cells [20] and enzyme expressed from recombinant DNA [21]. The inability to make 'de novo' methylation of ds DNA, that distinguishes *C. variopedatus* enzyme from all the others, cannot be attributed to possible factors influencing its activity because this property is consistently identical for samples at the centrifuged homogenate level and at the level of purified immunoprecipitated molecule.

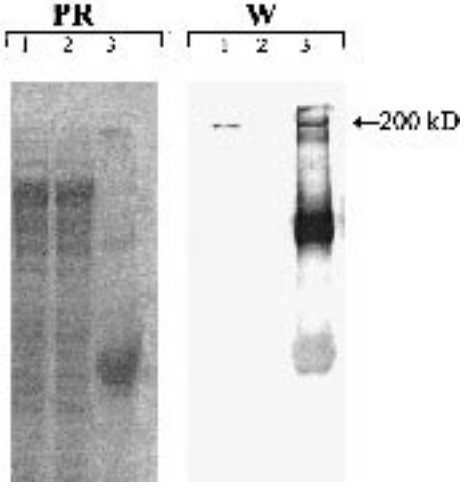

Fig. 3. Western blot of *C. variopedatus* embryos DNA-MTase with anti-sea urchin DNA-MTase antibodies. PR, blot of SDS-PAGE stained with Ponceau Red; W, same blot after immunoenzymatic reaction. Lane 1, proteins in the centrifuged homogenate of *C. variopedatus* embryos; lane 2, proteins in the supernatant of the same sample after the immunoprecipitation step; lane 3, proteins eluted from the immunoprecipitated pellet by SDS buffer. Note that the enzyme is immunodetected only in lanes 1 and 3 of the Western blot. In lane 3 the goat secondary antibodies detect also the subunits of the rabbit primary antibodies that are eluted from the pellet together with the enzyme by the SDS buffer.

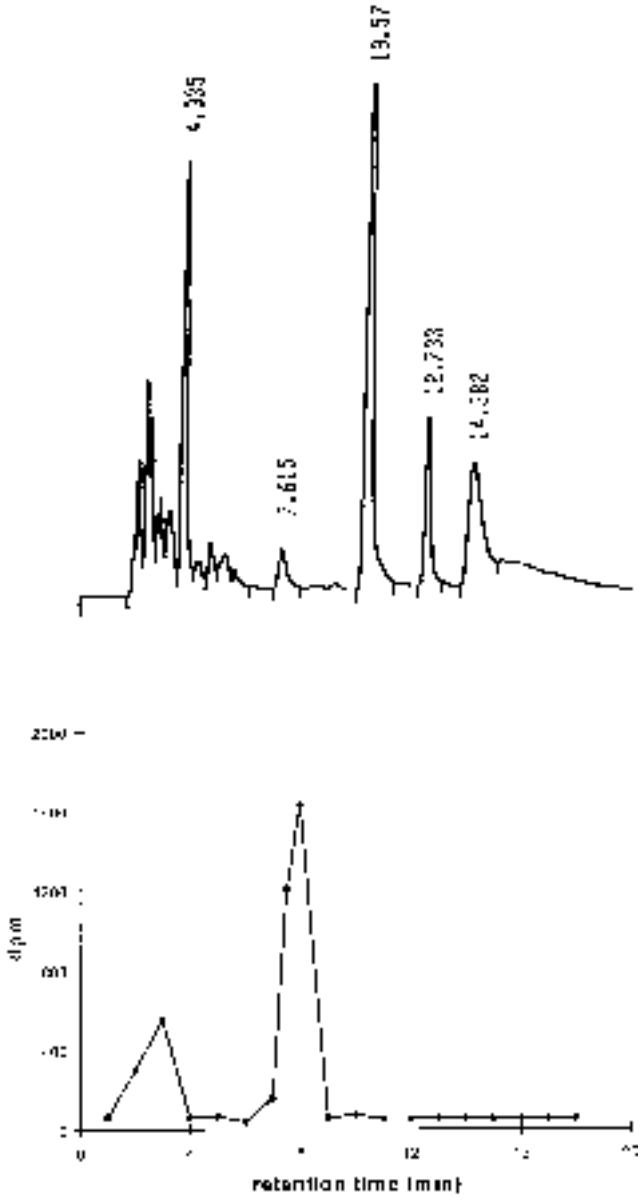

Fig. 4. HPLC analysis of the bases methylated on ss *M. luteus* substrate DNA by *C. variopedatus* enzyme. Upper panel: Elution pattern of the bases obtained by hydrolysis of substrate DNA after the enzymatic methylation. Lower panel: Radioactivity measurements on the fractions of the same column. The eluted bases have been identified as C at 4.03 min, m^5-C at 7.61 min, G at 10.57 min, A at 12.73 min and T at 14.38 min, respectively. The column was eluted at 1 ml/min. Fractions of 1 ml volume were collected except at 7.5 and 8 min where the volume was 0.5 ml.

3.2. Immunoidentification and molecular weight determination of *C. variopedatus* DNA-MTase

The Western blot of the enzyme in the centrifuged homogenate shows only one protein component with mobility value corresponding to about 200 kDa (Fig. 3, lane 1), similar to that of the sea urchin Dnmt1 [10]. The molecular weight of the enzyme in its active state was determined by gel filtration on a Sephadex G-100 column of the ammonium sulphate fraction prepared from the centrifuged homogenate. The activity is eluted in a volume corresponding to values between 150 and 200 kDa (data not shown) indicating that the about 200 kDa molecular weight protein observed by Western blots of SDS-PAGE of embryonic extracts is that of the active enzyme.

3.3. Identification of the base methylated by the enzyme in the substrate DNA

The results of the HPLC fractionation of the bases hydrolyzed from the enzymatically methylated *M. luteus* ss DNA (Fig. 4, upper panel), show the pattern of elution of bases in the ratio expected for that DNA with the addition of m^5-C [5]. Determination of radioactivity values in the eluted fractions demonstrates that the incorporated radioactive methyl groups are present only in the peak corresponding to m^5-C (Fig. 4, lower panel). The methylated cytosines, not present in the initial substrate, could only derive from the enzymatic activity that transferred radioactive methyl groups from SAM to DNA.

4. Conclusions

The data presented here are the first description of the properties of a DNA-MTase in an invertebrate organism such as the marine annelid worm *C. variopedatus*. The molecular weight value, the immunoreactivity and the catalytic properties of the molecule indicate that it is a Dnmt1 type enzyme with the unique property to be unable to methylate ds DNA. This suggests that the enzyme present in the ancient organisms acquired the ability to methylate 'de novo' ds DNA in the line eventually evolving to vertebrates. Such possibility is in agreement with the suggestion that the ability of Dnmt1 enzymes to methylate ds DNA might have relevance in the development of the large variety of cell types and differentiated tissues that are typical of the organisms of the vertebrate line.

Acknowledgements: This work was supported in part by 40% funds of the Italian MURST and in part by CNR contract N. 98.01108 CT14.

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