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Conserved sequence motif DPPY in region IV of the phage T4 Dam DNA-[N^6-adenine]-methyltransferase is important for S-adenosyl-L-methionine binding

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ABSTRACT

Comparison of the deduced amino acid sequences of DNA-[N^6-adenine]-methyltransferases has revealed several conserved regions. All of these enzymes contain a DPPY-motif, or a variant of it. By site-directed mutagenesis of a cloned T4 *dam* gene, we have altered the first proline residue in this motif (located in conserved region IV of the T4 Dam-MTase) to alanine or threonine. The mutant enzymic forms, P172A and P172T, were overproduced and purified. Kinetic studies showed that compared to the wild-type (wt) the two mutant enzymic forms had: (i) an increased (6 and 23-fold, respectively) K_m for substrate, S-adenosyl-methionine (AdoMet) and an increased (6 and 23-fold) K_i for product, S-adenosyl-homocysteine (AdoHcy); (ii) a slightly reduced (1.5 and 3-fold lower) k_{cat}; (iii) a strongly reduced k_{cat}/K_m^{AdoMet} (10 and 80-fold); and (iv) the same K_m for substrate DNA. Equilibrium dialysis studies showed that the mutant enzymes had a reduced (3 and 7-fold lower) K_a for AdoMet; all forms bound two molecules of AdoMet. Taken together these data indicate that the P172A and P172T alterations resulted primarily in a reduced affinity for AdoMet. This suggests that the DPPY-motif is important for AdoMet-binding, and that region IV contains an AdoMet-binding site.

INTRODUCTION

DNA methyltransferases (MTases) recognize specific nucleic acid sequences as their targets and transfer the methyl group from AdoMet to adenine or cytosine residues. MTases that are components of bacterial restriction–modification systems protect the cellular DNA against degradation by methylating specific bases within the sequence recognized by the cognate restriction enzyme. There are three major classes of MTases that differ in the resulting product of modification introduced: viz. N^6-methyladenine (m^{6}A), N^4-methylcytosine (m^{4}C) or C5-methylcytosine (m^{5}C). Although the amino acid (aa) sequences have been determined for more than 90 MTases (1), no X-ray crystallographic structure is available and little is known about structure–function relationships in these enzymes. The primary structures of MTases have been investigated in some detail (2–4). All of the m^{6}A-MTases contain the sequence Asp/Asn-Pro-Pro-Tyr (D/N-P-P-Y), first noted in region IV of the phage T4 and *E.coli* Dam-MTases by Hattman et al. (5). This suggested a common function for this region, such as the substrate (AdoMet)-binding site or a catalytic site (6,7,8). It was shown that FokI MTase has two functional domains, each containing a DPPPY-motif; and mutation of this motif caused loss of enzyme activity (9). However, no biochemical experiments directly focussing on the function of this motif has been reported.

We have investigated the function of region IV in the phage T4 Dam-MTase by introducing mutations in the DPPY-motif. Alteration of the first proline residue (Pro-172) to alanine or threonine changed primarily the affinity of the enzyme for AdoMet. This suggests that the DPPY-motif is important for AdoMet-binding, and that region IV contains an AdoMet-binding site.

MATERIALS AND METHODS

Chemicals and resins

[^{3}H]-AdoMet (73 Ci/mmol) was from New England Nuclear and unlabeled AdoMet (90% pure) was from Sigma. Phosphocellulose and DE81 ion-exchange filter paper were purchased from Whatman. Hydroxyapatite was from BioRad.

Mutagenesis

The *Eco*RI–*Bam*HI fragment from pSSH-12 (10) was transferred into a derivative of pGC1, which lacked the *Xba*I site, to form pGC712. The codon for aa 163 was altered from TTA (Leu) to CTA (Leu) by site-directed mutagenesis, thereby creating an *Xba*I site just upstream of region IV. Mutagenesis was performed by a slight modification of the method of Kunkel, et al. (11); viz. Sequenase (US Biochemical) replaced *E.coli* PolIk and only one primer was used. The new plasmid, designated pGC712-*Xba*I, was then mutagenized to obtain mutants at Pro-172 in region IV.

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Bacterial growth

Escherichia coli GM 2971 [F⁻*mrr*⁻ *hsd*S20 $r_B^-m_B^-$ *ara*14 *pro* A2 *lac* Y1 *gal* K2 *rsp*L20 (*str* r) *xyl* 5 *mtl*1 *sup*E44 *dam*13::Tn9 Cmr] was from M.G.Marinus. Cells containing an appropriate plasmid were grown in ampicillin-containing LB broth (12) at 30°C to an OD^{600} = 0.8 to 1.0. The temperature was raised to 42°C and incubation continued an additional 3 hrs. The cells were collected by low-speed centrifugation and stored frozen at −20°C.

Purification of wt and mutant T4 Dam-MTases

A detailed procedure describing the purification will be published elsewhere (Kossykh, Schlagman and Hattman, manuscript in preparation). In brief, frozen cells were thawed and suspended in 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 7 mM 2-mercaptoethanol (=PEM buffer), containing 0.4 M NaCl, 0.1% NP-40 and 100 μl phenylmethylsulfonyl fluoride. Cells were disrupted by sonication and cellular debris was removed by centrifugation at 100,000g for 1.5 hrs. The supernatant was diluted two-fold in PEM buffer and applied to a phosphocellulose column. Fractions containing Dam activity were pooled and chromatographed on an hydroxyapatite column.

Kinetic determinations

Apparent K_m and k_{cat} values for AdoMet and DNA were determined by monitoring ^3H-CH_3 transfer from labeled AdoMet to substrate DNA (unglucosylated, unmethylated T4 *gt*⁻ *dam*⁻). Reaction mixtures (50 μl) contained 100 mM Tris−HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 200 μg per ml of bovine serum albumin and variable concentrations (see Table II) of enzyme, DNA, AdoMet and AdoHcy. Under these conditions, methyl transfer was linear for at least 30 min. After incubation at 37°C for 10 min, 40 μl were removed and spotted onto DE81 filters which were washed and counted in a scintillation spectrometer. The moles of ^3H-CH_3 label transferred to DNA was calculated from the radioactivity bound to the filter and the [^{3}H]-AdoMet-specific activity. All kinetic data were analyzed using the 'Enzyme Kinetics' program from Trinity Software.

Binding of AdoMet

Binding of AdoMet to the wt and mutant Dam forms was determined by equilibrium dialysis (13). In an apparatus containing eight sets of dialysis cells, the two chambers of each dialysis cell were separated by a membrane (M_r 6000−8000 cutoff). The enzyme (1−5 μM) in 300 μl of 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 7 mM 2-mercaptoethanol and 0.1 M NaCl was placed on one side of the membrane; an equal volume of solution containing [^{3}H]-AdoMet was placed on the other side. The two solutions were allowed to equilibrate by gently rocking the apparatus for 48 hrs at 4°C. The amount of AdoMet bound to the enzyme was calculated from the difference in radioactivity measured on the two sides of each dialysis cell and from the specific activity of the [^{3}H]-AdoMet. In some experiments, [^{3}H]-AdoMet was added at the same concentration on both sides of the membrane.

Other analytical procedures

SDS-polyacrylamide gel electrophoresis was carried out according Laemmli (14). Protein concentrations were determined routinely by the method of Bradford (15) with bovine serum albumin as the standard; however, amino acid composition analysis was used to determine the protein concentration for the AdoMet-binding studies. Purity of commercial unlabeled AdoMet was confirmed by thin-layer chromatography on Machary-Nagel CEL 300 sheets in 66% ethanol.

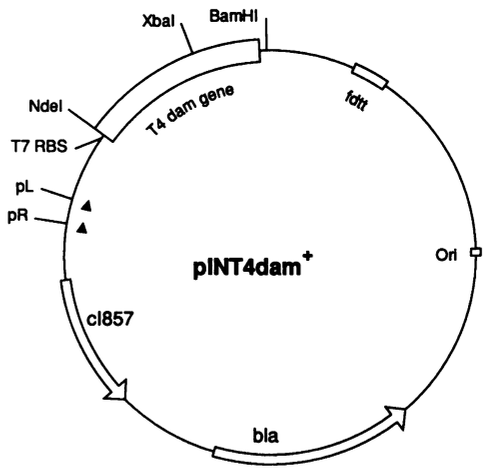

Figure 1. Schematic diagram of plasmid pINT4*dam*⁺. The vector used to create pINT4*dam*⁺ was pJW2 (8). The arrows indicate the direction of transcription of the *cI*857 allele, the *bla* gene and the λpR and λpL promotors contained in the vector. RBS is the ribosomal-binding-site for the phage T7 gene10, and tt is a phage fd transcription-terminator. The *NdeI*−*XbaI* fragment of T4 *dam* gene was derived from plasmid pSSH-17 (8) following two cycles of site-directed mutagenesis. One cycle altered the codon at aa 163 from TTA [Leu] to CTA [Leu], creating an *XbaI* site just upstream of region IV. The other cycle created an *NdeI* site at the AUG [Met] start of the gene. The*XbaI*−*Bam*HI fragment of the T4 *dam* gene came from plasmid pSSH-12 (8) following site-directed-mutagenesis to create an *XbaI* site [without altering aa coding] just upstream of region IV.

Table I. Activity in cell extracts of wt and mutant T4 Dam-MTases

Enzyme	% activity
wt	100
P172A	8.0
P172T	1.5
P172R	1.0
P172C	<1.0

Cell extracts were prepared as described in Materials and Methods. Standard asssay reaction mixtures (50 μl) contained 100 mM Tris−HCl, pH8.0, 1mM EDTA, 1 mM DTT, 200 μg per ml bovine serum albumin, 25 μg per ml T4 *gt*⁻ *dam*⁻ DNA, 0.5 μM AdoMet and different dilutions of cell extracts. 100% of activity corresponds to 200 pmol ^3H-CH_3 groups transferred/mg/min.

RESULTS

Production of aa replacements at Pro-172

We used oligonucleotide site-directed mutagenesis of the *XbaI*−*Bam*HI fragment to change Pro-172, which is located in the region IV DPPY-motif (9). Following DNA sequence analysis by the method of Sanger et al. (16) the *XbaI*−*Bam*HI fragments containing the mutations were subcloned into the corresponding sites of plasmid pINT4*dam*⁺ (see Fig.1). The standard MTase assay (Material and Methods) was then used to determine Dam activity in crude extracts containing mutant enzymic forms. The

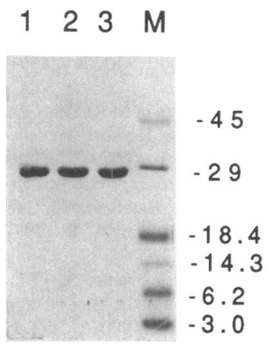

Figure 2. SDS-polyacrylamide gel electrophoresis of wt and mutant MTases. Purified MTases were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Lanes 1−3 contain purified wt, P172A, and P172T MTases, respectively. Lane 4 contains a mixture of molecular mass markers including ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa) and insulin (3.0 kDa).

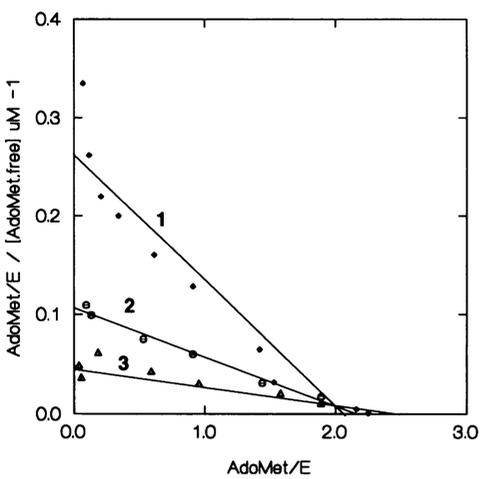

Figure 3. Scatchard plot of [^{3}H]-AdoMet binding to the T4 Dam-MTase measured by equilibrium dialysis. 0.3−1.5 μmol of MTase (total volume of 300 μl) was introduced into the left chamber and [^{3}H] AdoMet at various concentrations (total volume of 300 μl) was introduced into the into right chamber. After 48 hrs at 4°C with continuous rocking, 100 μl was removed from each chamber and the radioactivity was measured. The specific activities were used to calculate the moles of AdoMet bound, and the data were plotted according to the equation: $A_b/[A_f] = K_a(n-A_b)$, where A_b is the number of moles AdoMet bound per mole of MTase, $[A_f]$ is the molar concentration of unbound AdoMet, Ka is the association constant, and n is the number of binding sites per enzyme molecule (16). The points are the means of at least 3 independent experiments;and graphs were determined by linear regressinto the mean.1=wt; 2=P712A; 3=P172T.

results summarized in Table I show that each of the four isolated substitutions resulted in sharply reduced MTase activity under standard assay conditions. Two of the replacements, P172A and P172T, were studied further. The wt and mutant T4 Dam-MTases were overproduced and purified, as described in the Materials and Methods. A homogeneous protein of about 30 kDa was observed (Fig.2), consistent with the predicted size deduced from the T4 *dam* gene open reading frame (9) and from the observed molecular weight of the native enzyme (unpublished). Milligram quantities of apparently homogeneous protein from the wt and mutants were purified from 3 liters of cell culture (Fig.2).

Steady state kinetic analysis

Table II summarizes the results of steady state kinetic analyses. The k_{cat} and apparent K_m's for AdoMet and DNA were derived from initial velocity experiments by varying AdoMet and DNA concentrations. The results show that, compared to the wt, the K_m for DNA was unchanged in the mutant forms, and the k_{cat} was only slightly (1.5 to 3-fold) reduced. In contrast, the K_m for AdoMet and K_i for AdoHcy were 6 to 23-fold higher for the mutants, suggesting that the Pro-172 mutations specifically affected the affinity for AdoMet. It should be noted that in the standard assay the AdoMet concentration is at or below the K_m of the two mutant enzymes; that accounts in part for the reduced activity observed in Table I.

Equilibrium dialysis binding of AdoMet

In view of the increased K_m values for AdoMet, we determined the association constant by equilibrium dialysis (see Materials and Methods). The results of a Scatchard analysis (17) and direct plot methods are summarized in Fig.3 and Table II; we observed that at saturation two moles of AdoMet were bound per mole of native monomers of wt and mutant Dam-MTase. Under the experimental conditions used the mutant enzymes P172A and P172T had about 3-fold and 7-fold lower K_a values for AdoMet, respectively, than the wt enzyme. These results confirm that the mutations reduced AdoMet-binding ability.

DISCUSSION

The primary structure of a number of MTases has been deduced. DNA MTases (m^5C, m^4C, m^6A) are characterized by the presence of two highly conserved motifs (8). The AdoMet-binding sites of the *Eco*RII-MTase (a m^5C-MTase) (18) and the *Eco*RI-MTase (an m^6A-MTase) (19) were probed by photoaffinity labeling with AdoMet and 8-azido-AdoMet, respectively. In the case of the *Eco*RII MTase the major photoreaction product, S-methyl cysteine, was produced in one of the conserved regions common to m^5C-MTases. In contrast, the photolabeled region of the *Eco*RI-MTase was not located in

Table II. Catalytic and substrate binding parameters of wt and mutant T4 Dam-MTases

MTase	k_{cat} (sec^{-1})	K_m [DNA] (10^{-12} M)	K_m [AdoMet] (10^{-6} M)	k_{cat}/K_mAdoMet (10^6 $M^{-1}sec^{-1}$)	K_a [AdoMet] (10^5 M^{-1})
wt	0.13	0.9	0.09	1.44	1.31
P172A	0.08	1.2	0.50	0.16	0.50
P172T	0.04	1.3	2.20	0.018	0.20

The apparent steady state kinetic parameters were obtained at 37°C with 0.36−10.0 pM T4 *gt*$^-$ *dam*$^-$ DNA, 0.05−5.0 μM AdoMet in 100 mM Tris, pH 8.0, 1 mM EDTA, 200 μg per ml bovine serum albumin,1 mM DTT and 0.3 or 0.6 nM MTase. Association constants (K_a) were determined from equilibrium dialysis studies at 4°C using 1−5 μM MTase and 0.10 to 20 μM [^{3}H]-AdoMet in 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1 M NaCl.

any of the conserved sequences of m^6A-MTases. In T4 Dam the two conserved motifs identified in m^6A-MTases are at aa positions 28–40 (region I) and 164–179 (region IV)(5). In our study, site-directed mutagenesis was performed to produce mutant proteins in which Pro-172 [part of the highly conserved DPPY-motif in region IV] was replaced by Ala or Thr. The corresponding mutant proteins, P172A and P172T, were overexpressed and purified. They were not markedly different from the wt enzyme with respect to k_{cat} or K_m for substrate DNA (Table I). In contrast, however, the P172 substitutions resulted in a 6 to 23-fold increase in K_m and a 3 to 7-fold decrease in K_a for AdoMet. This indicates that the mutations affected primarily AdoMet-binding.

Pro-172 is one of four highly conserved aa located in a region predicted to form a finger-like structure containing a pleated sheet and two turns (P-P) followed by another pleated sheet (7). Pro-172, however, does not appear to have to directly contact AdoMet, because the mutated forms are still functional. Our data also show that AdoMet binds the T4 Dam-MTase at two sites. In the case of the related *Eco*dam-MTase (20), the existence of two AdoMet-binding sites has been postulated, one having an allosteric role and the second being catalytic. Fluorescence spectroscopy studies (data not shown) indicate that a T4 Dam conformational change does indeed occur following AdoMet-binding. The region IV mutants also display this conformational change, but only at much higher AdoMet concentrations than for the wt Dam. Therefore, we suggest that region IV contains the allosteric AdoMet-binding site. The slight change observed in the k_{cat} might reflect an influence of the allosteric site on AdoMet-binding in the catalytic site, or that AdoMet is transferred from the allosteric to the catalytic site. Folding of the T4 Dam-MTase could bring regions I and IV in close proximity to create the active site in which both binding and catalytic functions are carried out. Answers to these questions await detailed high resolution structural analysis of the protein.

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REFERENCES

1. Wilson, G. G. (1991) Nucl. Acids Res. **19,** 2539–2565.
2. Chandrasegaran, S. and Smith, H. O. (1988) *In* Sarma, M. H. and Sarma, R. H. (eds.) Structure and Expression. Adenine Press, Guilderland, NY, Vol.1, pp.149–156.
3. Pósfai, J., Bhagwat, A. S., Pùsfai, G. and Roberts, R. J. (1989) Nucleic Acids Res. **17,** 2421–2435.
4. Lauster, R.,Trautner,T. A. and Noyer-Weider, M. (1989) J. Mol. Biol. **206,** 305–312.
5. Hattman, S., Wilkinson, J., Swinton, D., Schlagman, S., MacDonald, P. M. and Mosig, G. (1985) J. Bacteriol. **164,** 932–937.
6. Lauster, R., Kriebardis, A. and Guschlbauer, W. (1987) FEBS Lett. **220,** 167–176.
7. Guschlbauer, W. (1988) Gene **74,** 211–214.
8. Klimasauskas, S., Timinskas, A., Menkevicius, S., Butkiene, D., Butkus, V. and Janulaitis, A. (1989) Nucl. Acids Res. **17,** 9823–9832.
9. Sugisaki, H., Kita, K., and Takanami, M. (1989) J. Biol. Chem. **264,** 5757–5761.
10. Schlagman, S. L., Miner, Z., Fehér, Z. and Hattman, S. (1988) Gene **73,** 517–530.
11. Kunkel, T. A., K. Bebenek, and J. McClary. 1991. Methods Enzymol. **206,** 125–139.
12. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Teraoka, H. and Nierhaus, K. H. (1979) Methods Enzymol. **59,** 862–866
14. Laemmli, U. K. (1970) Nature **227,** 680–685.
15. Bradford, M. M. (1976) Anal.Biochem. **72,** 248–254.
16. Sanger, F. Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA **74,** 5463–5467.
17. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. **51,** 660–672.
18. Som, S. and Friedman, S. (1991) J. Biol. Chem. **266,** 2937–2945.
19. Reich, N. O. and Everett, E. A. (1990) J. Biol. Chem. **265,** 8929–8934.
20. Bergerat, A. and Guschlbauer, W. (1990) Nucl. Acids Res. **18,** 4368–4375.