Conserved sequence motif DPPY in region IV of the phage T4 Dam DNA-[N⁶-adenine]-methyltransferase is important for S-adenosyl-L-methionine binding

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ABSTRACT

Comparison of the deduced amino acid sequences of DNA-[N⁶-adenine]-methyltransferases has revealed several conserved regions. All of these enzymes contain a DPPY [or closely related] motif. 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 [5 and 20-fold, respectively] K_m for substrate, S-adenosyl-methionine [AdoMet]; (ii) a slightly reduced [2 and 4-fold lower] k_{cat} ; (iii) a strongly reduced k_{cat}/K_m AdoMet [10 and 100-fold]; and (iv) almost the same K_m for substrate DNA. Equilibrium dialysis studies showed that the mutant enzymes had a reduced [4 and 9-fold lower] K_a for 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 or is part of an AdoMet-binding site.

INTRODUCTION

DNA methyltransferases [MTases] recognize specific nucleic acid sequences as their targets and transfer the methyl group from AdoMet to an adenine or cytosine residue. 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, which differ in the resulting product of modification introduced: viz. N⁶-methyladenine [m⁶A], N⁴-methylcytosine [m⁴C] or C5-methylcytosine [m⁵C]. 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 m⁶A-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 (6–8). It was shown that FokI MTase has two functional domains, each containing a DPPY-motif; and mutation of this motif caused loss of enzyme activity (9). However, no biochemical experiment 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 or is part of an AdoMet-binding site.

MATERIALS AND METHODS

Chemicals and resins

[³H]-AdoMet (73 Ci/mmol and 15 Ci/mmol) was from New England Nuclear. Unlabeled AdoMet (90% pure; from Sigma) was purified by HPLC on a C₁₈-reversed-phase column [25 cm by 4.6 mm, fitted with a 4.5 cm by 4.6 mm guard C₁₈-column] using isocratic elution in 50 mM ammonium acetate, pH 4.5, with 5% methanol. The AdoMet was dried in vacuo, dissolved in 5 mM HCl, 5% ethanol and stored at –20°C [or at 4°C if it was used within one week]. The final preparation was 99.6% pure. The concentration of AdoMet was calculated using a molar extinction coefficient at 256 nm of 14,700 [in acid] (10). Phosphocellulose and DE81 ion-exchange filter paper were purchased from Whatman. Hydroxyapatite was from BioRad.

Mutagenesis

The *EcoRI*-*Bam*HI fragment from pSSH-12 (11) was transferred into a derivative of pGC1, which lacked the *Xba*I site, to form pGC712. The codon for aa 163, in region IV, 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.* (12); viz., Sequenase (U. S. 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.

Bacterial growth

Escherichia coli GM 2971 [F^- *mrr*⁻ *hsdS*20 *r*_B-*m*_B- *ara*14 *pro* A2 *lac* Y1 *gal* K2 *rsp*L20 (*str*^r) *xyl* 5 *mut*1 *sup*E44 *dam*13::Tn9 Cm^r] was from M.G. Marinus. Cells containing an appropriate plasmid were grown in ampicillin-containing LB broth (13) at 30°C to an OD₆₀₀ = 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 (Kosykh, 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% NP40 and 100 μ l 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 purified to apparent homogeneity on an hydroxyapatite column.

Kinetic determinations

Apparent K_m [DNA], K_m [AdoMet] and k_{cat} values were determined by monitoring [³H]-CH₃ transfer from labeled AdoMet to substrate DNA (unglucosylated, unmethylated T4 *gt*⁻ *dam*⁻). Reaction mixtures [50 μ l] contained 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 200 μ g per ml of bovine serum albumin and variable concentrations (see Table II) of enzyme, DNA or AdoMet. Under the conditions employed, methyl transfer was linear for at least 30 min. After incubation at 37°C for 10 min, 40 μ l was removed and spotted onto DE81 filters, which were then washed three times with 50 mM KH₂PO₄, twice with 80% ethanol, once with 95% ethanol, dried and the radioactivity counted in a liquid scintillation spectrometer. The number of methyl groups transferred to the DNA was calculated from the radioactivity bound to the filter [minus that in the zero time aliquot] divided by the specific activity. Kinetic parameters were obtained from a plot of $V/[S]$ vs $[S]$ 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 (14). 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 μ M] in 300 μ l of 40 mM potassium phosphate, pH 7.4, 1 mM EDTA and 0.1 M NaCl was placed on one side of the membrane; an equal volume of solution containing 1 to 200 μ M [³H]-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 [³H]-AdoMet. 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, K_a is the association constant, and n is the number of binding sites per enzyme molecule (14,15). In some experiments, [³H]-AdoMet was added at the same concentration on both sides of the membrane. Purity of AdoMet before and after dialysis was confirmed by HPLC analysis, and it indicated that less than 10% of AdoMet degraded during dialysis.

Other analytical procedures

SDS-polyacrylamide gel electrophoresis was carried out according Laemmli (16). Protein concentrations were determined routinely by the method of Bradford (17) with bovine serum albumin as the standard; however, amino acid composition analysis was used to determine the protein concentration for the AdoMet-binding studies.

RESULTS

Production of aa replacements at Pro-172

We used oligonucleotide site-directed mutagenesis of the *Xba*I-*Bam*HI fragment to change Pro-172, which is located in the region IV DPPY-motif (5). Following DNA sequence analysis by the method of Sanger *et al.* (18) the *Xba*I-*Bam*HI fragments containing the mutations were subcloned into the corresponding sites of plasmid pINT4 dam^+ (see Fig.1). The standard MTase assay (Materials and Methods) was then used to determine Dam activity in crude extracts containing the wt or mutant enzymic form. The results summarized in Table I show that each of the four isolated substitutions resulted in sharply reduced MTase activity. 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

Figure 1. Schematic diagram of plasmid pINT4 dam^+ . The vector used to create pINT4 dam^+ was pJW2 (30). The arrows indicate the direction of transcription of the *cl857* allele, the *bla* gene and the λ pR and λ pL promoters contained in the vector. RBS is the ribosomal-binding-site for the phage T7 gene10, and it is a phage fd transcription-terminator. The *Nde*I-*Xba*I fragment of T4 *dam* gene was derived from plasmid pSSH-17 (11) following two cycles of site-directed mutagenesis. One cycle altered the codon at aa 163 from TTA [Leu] to CTA [Leu], creating an *Xba*I site just upstream of region IV. The other cycle created an *Nde*I site at the AUG [Met] start of the gene. The *Xba*I-*Bam*HI fragment of the T4 *dam* gene came from plasmid pSSH-12 (11) following site-directed-mutagenesis to create an *Xba*I site [without altering aa coding] just upstream of region IV.

Methods. Milligram quantities of apparently homogeneous Dam from the wt and mutants were purified from 3 liters of cell culture (Fig.2). The protein was about 30 kDa (Fig.2), consistent with the predicted size deduced from the T4 *dam* gene open reading frame (11).

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 [2 to 4-fold] reduced. In contrast, the K_m for AdoMet was 5 to 20-fold higher for the mutants, suggesting that the Pro-172 mutations specifically affected the affinity for AdoMet. We also observed a comparable increase in the K_i for the methylation product, S-adenosyl-homocysteine [data not shown], which is a competitive inhibitor of 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

Because the mutant enzymic forms exhibited an increased K_m for AdoMet, we determined the association constant by equilibrium dialysis (see Materials and Methods). The results of a Scatchard analysis (15) and direct plot methods are summarized in Table II; we observed that at saturation the binding stoichiometries were 1.35 ± 0.27 for the wt, and 1.21 ± 0.25 and 1.37 ± 0.21 for the mutant enzymes, P172A and P172T, respectively. Under the experimental conditions, P172A and P172T had about 4-fold and 9-fold lower K_a values for AdoMet, respectively, than the wt enzyme. These results confirm that the mutations reduced the AdoMet-binding ability.

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].

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 assay reaction mixtures [50 μ l] contained 100 mM Tris-HCl, pH8.0, 1m M EDTA, 1 mM DTT, 200 μ g per ml bovine serum albumin, 25 μ g per ml T4 *gt⁻ dam⁻* DNA, 0.5 μ M AdoMet and different dilutions of cell extracts. 100% of activity corresponds to 200 pmol [^{3} H]-CH₃ groups transferred/mg/min.

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

MTase	k_{cat} (sec^{-1})	K_m [AdoMet] (10^{-6} M)	$k_{cat}/K_m^{\text{AdoMet}}$ ($10^9 \text{ M}^{-1} \text{sec}^{-1}$)	K_m [DNA] (10^{-12} M)	K_a [AdoMet] (10^5 M^{-1})
wt	0.15 (± 0.02)	0.10 (± 0.02)	1.32 (± 0.35)	1.14 (± 0.40)	0.37 (± 0.11)
P172A	0.07 (± 0.04)	0.53 (± 0.23)	0.13 (± 0.05)	1.65 (± 0.50)	0.10 (± 0.02)
P172T	0.04 (± 0.02)	2.05 (± 0.86)	0.013 (± 0.004)	1.84 (± 0.36)	0.04 (± 0.01)

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 μ M AdoMet in 100 mM Tris, pH 8.0, 1 mM EDTA, 200 μ g per ml bovine serum albumin, 1 mM DTT and 0.3 or 0.6 nM MTase. Association constants [K_a] were determined by Scatchard analysis of the results of equilibrium dialysis [see Materials and Methods]. The kinetic and equilibrium constants are the mean values (± 1 S. D.) from at least three independent experiments.

DISCUSSION

From the deduced primary structure of a fairly large number of DNA-[m⁵C, m⁶C, m⁶A]-MTases the presence of highly conserved sequence motifs has been recognized (2–4,8). A sequence resembling the LFXGxGG motif of the m⁵C-MTases is found in most all MTases (19). The identification of this motif in RNA- and protein-MTases, which all use AdoMet as the methyl group donor (20), has led to the speculation that it is the AdoMet-binding site. Another motif in m⁵C-MTases, DxxxGxPCQxxSxxG, is highly conserved and is thought to be the active [catalytic] site (21). There is evidence that the Cys in this motif forms a covalent bond with the C6-carbon of DNA-cytosine (22–24).

The m⁶C- and m⁶A-MTases share another conserved motif, (D,N,S)PP(Y,F). From similarities in the sequences flanking this motif with those flanking the PCxxxS motif of m⁵C-MTases, it was inferred that the former has an analogous function; viz., methylation of the exocyclic amino group of the target base (8).

The importance of the presence of the (D,N,S)PP(Y,F) motif is illustrated by the unusual type IIs FokI-MTase, which methylates both complementary strands of its asymmetric target sequence. In this MTase, the two motifs occur twice in the the polypeptide chain. Site-directed mutagenesis of the FokI-MTase gene causing a change in either of the (D,N,S)PP(Y,F) motifs

destroyed the capacity of the enzyme to methylate one of the asymmetric target sites (9). These data showed that the (D,N,S)PP(Y,F) motif is crucial for enzyme activity, but the precise role of this motif in methylation has not been determined.

Photoaffinity labeling experiments with radioactive AdoMet or azido-AdoMet have attempted to determine the peptide involved in AdoMet-binding by the *EcoRI*, *EcoDam* and *EcoRII* MTases (25–27). None of these experiments gave definitive proof that any of the motifs was directly involved in AdoMet-binding. However, *EcoRII*-MTase peptides containing Cys in the motif, DxxxGxPCQxxSxG, were cross-linked to AdoMet. This could be taken as an indication that the AdoMet-binding site is in close proximity to the catalytic site.

In our study with the T4 Dam-MTase, site-directed mutagenesis was performed to produce mutant proteins in which Pro-172, the first Pro in the (D,N,S)PP(Y,F)-motif [present as DPPY 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 II). In contrast, however, these substitutions resulted in 6 to 23-fold increase in K_m and 3 to 7-fold decrease in K_a for AdoMet. This indicates that the mutations primarily affect AdoMet-binding.

Our preliminary spectroscopic results measuring tryptophan fluorescence quenching indicate that T4 Dam-MTase undergoes a conformational change following AdoMet-binding. The Pro-172 mutants also display this conformational change, but only at much higher AdoMet concentrations than the wt enzyme. This is consistent with an alteration in affinity for AdoMet.

The results of equilibrium dialysis showed that one AdoMet is bound per 30 kDa T4 Dam monomer equivalent. NMR studies suggested a dual role for AdoMet, and two AdoMet-binding sites have been postulated for the *EcoDam*-MTase, one allosteric and one catalytic (28, 29). It remains to be seen whether AdoMet plays such a dual role in other MTases.

Finally, we believe that Pro-172 does not have to make a stereospecific contact with AdoMet; this follows from the fact that the P172A and P172T MTases are still active at high AdoMet concentration. This suggests that Pro-172 has an important structural function critical for enzyme activity. The role of the other amino acids within and flanking the motif also remains unknown; but, it is likely that region IV of T4 Dam contains or is part of the AdoMet-binding site. Answers to these questions await detailed high resolution structural analysis of the protein.

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