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Short communication

Recombinant α and β Subunits of M.*Aqu*I Constitute an Active DNA Methyltransferase

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***Aqu*I DNA methyltransferase, M.*Aqu*I, catalyses the transfer of a methyl group from S-adenosyl-L-methionine to the C5 position of the outermost deoxycytidine base in the DNA sequence 5'CYCGRG3'. M.*Aqu*I is encoded by two overlapping ORFs (termed α and β) instead of the single ORF that is customary for Class II methyltransferase genes. The structural organization of the M.*Aqu*I protein sequence is quite similar to that of other bacterial C5-DNA methyltransferases. Ten conserved motifs are also present in the correct order, but only on two polypeptides. We separately subcloned the genes that encode the α and β subunits of M.*Aqu*I into expression vectors. The overexpressed His-fusion α and β subunits of the enzyme were purified to homogeneity in a single step by Nickel-chelate affinity chromatography. The purified recombinant proteins were assayed for biological activity by an *in vitro* DNA tritium transfer assay. The α and β subunits of M.*Aqu*I alone have no DNA methyltransferase activity, but when both subunits are included in the assay, an active enzyme that catalyses the transfer of the methyl group from S-adenosyl-L-methionine to DNA is reconstituted. We also showed that the β subunit alone contains all of the information that is required to generate recognition of specific DNA duplexes in the absence of the α subunit.**

Keywords: Bacterial methylation, DNA binding, Protein expression and purification, DNA Methyltransferase.

DNA methyltransferases (MTases) are found in both eukaryotes and prokaryotes (Adams, 1985). In bacteria, methylation is a part of the Restriction-Modification (R-M) systems. All of the R-M systems are comprised of two sequence-specific enzymatic activities - a restriction endonuclease activity that cleaves DNA at a specific site, and a modification methyltransferase activity that recognizes the same target sequence as the cognate restriction activity within which it methylates a specific base. DNA from cells that contain such a system are usually fully modified and protected from digestion; whereas, foreign DNA that lacks the specific modification is cleaved on entry into the cell (Kuhnlein and Arber, 1972). R-M systems, therefore, appear to defend the cell from invasion by foreign DNA, usually in the form of viruses. They can, therefore, act as a primitive immune system (Price and Bickle, 1986).

DNA MTases can be classified into three groups (mC4, mA6, mC5), based on their sites of methylation. However, 5-methylcytosine is the only methylated base that is found in vertebrate and plant DNA (Razin and Riggs, 1980). C5-DNA MTases have extensive similarities. A comparative analysis of known C5-DNA MTase sequences showed that these proteins share an ordered set of sequence motifs that alternate with non-conserved regions (Som *et al*., 1987). Up to ten motifs can be identified in the majority of known sequences, including the C terminal 500 amino acids of the eukaryotic CpG DNA MTase (Bestor *et al*., 1988; Lauster *et al*., 1989; Posfai *et al*., 1989). Six motifs are considered highly conserved (I, IV, VI, VIII, IX, and X) (Cheng *et al*., 1993). The variable region, located between the conserved motifs VIII and IX, shows the greatest heterogeneity in size, sequence, and composition. Within the variable region, a small subregion that is termed the Target Recognition Domain (TRD) is directly involved in sequence specific recognition. According to the crystal structure of two members of the C5-DNA Mtases, motif I is involved in cofactor binding, and motif IV contains a conserved Pro-Cys dipeptide, which is part of the catalytic site (Cheng *et al*., 1993; Klimasauskas *et al*., 1994; Reinisch *et al*., 1995).

A sequence specific DNA MTase from *Agmenellum quadruplicatum*, M.*Aqu*I, was first reported by Karreman *et al*. (1986). It has absolute requirements for S-adenosyl-L-

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methionine (AdoMet) as a methyl donor, and the outermost cytosine residue in the CYCGRG sequence is the acceptor for the methyl group. The M.*Aqu*I gene was isolated as a 1.2 kb DNA fragment, and the complete nucleotide sequence was determined (Karreman and De Waard, 1990). It is encoded by two partly overlapping ORFs (termed α and β). The structural organization of the M.*Aqu*I protein is quite similar to that of other bacterial C5-DNA MTases. The 10 conserved motifs are also present in the correct order, but only on two polypeptides. The α polypeptide constitutes most of the AdoMet binding site, and contains the catalytic cysteine (Cys81 in the M.*Hha*I structure). The β subunit contains the TRD, together with homology blocks IX and X. Although a typical C5-DNA MTase is encoded by a single ORF, another bacterial MTase M.*Eco*HK311 has also been reported to require two polypeptides for *in vitro* methylation of target DNA (Lee *et al.*, 1995 & 1996). Karreman and De Waard (1990) demonstrated that M.*Aqu*I is comprised of two polypeptide chains, which constitute an active MTase. However, purified subunits were not used in their experiments.

We subcloned the genes that encode the α and β subunits of M.*Aqu*I in order to facilitate the purification of the two subunits separately for *in vitro* methylation studies. We also addressed the question of whether both of the subunits are required for DNA recognition and binding, or if the binding is only mediated by the β subunit that contains the TRD.

In order to determine whether the α and β subunits of M.*Aqu*I act as a DNA MTase *in vitro*, we subcloned the genes into the bacterial expression vector, pET14b. Original plasmids, pSDαβ and pβ that harbor the α and β genes respectively, were kindly provided by Dr. Christian Karreman. We used these plasmids as templates for amplification of the α and β genes. The α (747 bp) and β (420 bp) PCR products were introduced into pET14b separately in frame with the His-tag sequence to construct recombinant plasmids pETα and pETβ. *E.coli* BL21 (DE3) pLysS cells were separately transformed with pETα and pETβ. The recombinant polypeptides were insoluble when overexpressed in *E. coli*, judged by the predicted molecular weight of the induced proteins (30,000 Da for Hisα and 18,000 Da for Hisβ) on SDS-PAGE (data not shown). The polypeptides were solubilized in 6 M urea. Purification was carried out under denaturing conditions. Hisα and Hisβ were purified in a single step by nickel-chelate affinity chromatography. Approximately 10 mg of the denatured His fusion protein was purified from 1 liter of induced culture.

The main problem that we encountered during this work was the aggregation and subsequent refolding of recombinant proteins when overexpressed in *E.coli*. Reportedly, this can be overcome by reducing the expression levels of a protein within the cell. A reduction in the temperature where induction is performed may serve to increase the level of soluble protein, possibly by reducing aggregation as a consequence of a lower level of expression. The modification enzyme, M.*Msp*I, that is expressed as a GST fusion (Taylor *et al.*, 1993), and a yeast pseudo, C5-DNA MTase pmt I$^+$, that is expressed as a His-tag fusion (Pinarbasi *et al.*, 1996), were solubilized *in vivo* by reducing the induction temperature from 37°C to 30°C, or below. In order to overcome the solubility problem with Hisα and Hisβ, the *E.coli* BL21 (DE3) cells that harbored the recombinant plasmids (pETα and pETβ) were grown at 37°C until the OD_{600} = 0.6, then induced at 30, 28, and 25°C. No detectable effect on solubility was observed.

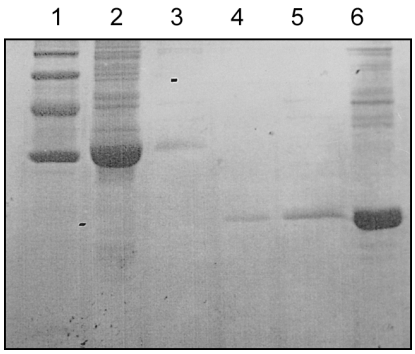

Fig. 1. SDS-PAGE analysis of the Hisα and Hisβ polypeptides after refolding. Protein expression from the T7 RNA polymerase promoter was induced by the addition of 0.1 mM IPTG for 3 h. Soluble and insoluble fractions of the extracts from the cells that contained pETα and pETβ were separated by centrifugation. The insoluble fraction was solubilized in 6 M urea for 3 h. Proteins were purified by Ni^{2+}-chelate affinity chromatography. The denatured material was refolded by stepwise dialysis. Aggregated material that was present in the dialysis solution was collected by centrifugation. The supernatants were analyzed on 15% SDS-PAGE. Lane 1: Molecular weight marker (97, 66, 45, 29 kDa), Lane 2: Insoluble fraction of the cells containing pETα after solubilization with urea, Lane 3: The Hisα polypeptide after purification and refolding, Lane 4, 5: The Hisβ polypeptide after purification and refolding, Lane 6: Insoluble fraction of the cells containing pETβ after solubilization with urea.

In order to refold the denatured proteins, a step-wise dialysis was performed. However, the proteins were found to re-precipitate upon removal of the urea. Dialysis was carried out under a range of conditions in order to raise the yield of recoverable active protein. However, it was discovered that both subunits have the tendency to aggregate at concentrations above 50 μM. Six molar urea solubilized both of the subunits, but after the removal of the denaturant most of the protein remained unfolded (Fig. 1).

Karreman and De Waard (1990) reported the molecular cloning and sequence analysis of M.*Aqu*I. Their result indicated that this enzyme comprises two polypeptides, termed α and β. However, the purified subunits were not used in their experiments. They examined the MTase activity of the enzyme by *in vivo* DNA protection assays after cloning both the α and the β ORFs in separate but compatible plasmid vectors. In their experiment, the transformation of *E.coli* with either plasmid that encoded the α or the β alone did not lead to the synthesis of a functional M.*Aqu*I. However, when the

Table 1. Tritiated methyl group transfer activity of M.*Aqu*I. All of the reactions were carried out in a total volume of 20 µl, including 1 µl of ^{3}HAdoMet (15 Ci/mmol), 1 µg of calf thymus DNA, 1 µM protein (0.5 mM of each protein was used when Hisα and Hisβ were assayed in the same reaction), and 2 µl of a 10X M.*Aqu*I reaction buffer (20 mM Tris-HCl pH: 7.9, 100 mM NaCl, 1 mM EDTA, 7 mM β-mercaptoethanol) for 1 h at 37°C. Following each of the incubations, the reaction mixtures were increased to 100 µl with the incubation buffer and loaded onto a spin column, then the fractions were collected. This process was repeated 6 times. The radioactivity in each fraction was counted for 1 min in 10 ml scintillation fluid. A control spin column with only DNA and ^{3}H AdoMet (no protein) was included in the assay in order to check the efficiency of the separation. Each assay was performed three times, and the average value was taken.

Protein	Fractions (c.p.m.)					
	1	2	3	4	5	6
No protein	47	53	184	722	2390	9954
Hisα	35	39	220	650	1500	11500
Hisβ	29	34	250	590	2000	11800
Hisα+Hisβ	5254	4321	380	447	1900	10250

two plasmids were introduced into *E.coli* together, the resulting transformed cells produced a functional M.*Aqu*I. In our experiments, we used both subunits in a purified form. Our *in vitro* MTase assay results support Karreman and De Waard's (1990) conclusions. As seen in Table 1, a proportion of radioactivity that was being eluted from the gel filtration column in the first two fractions shows that M.*Aqu*I incorporated ^{3}H methyl groups into calf thymus DNA when both the α and the β subunits were present in the reaction mixture. Separately assayed subunits were not as active as similar radioactivity counts that were obtained with the no-protein assay.

Primary sequence alignments between C5-DNA MTases have pointed to a major role for the hypervariable region of these enzymes in sequence specific DNA recognition. This was shown by domain-swapping experiments of multispecific MTases (Trautner *et al.*, 1988), as well as by crystal structures of monospecific MTases M.*Hha*I (Cheng *et al.*, 1993) and M.*Hae*III (Reinisch *et al.*, 1995). The structure of M.*Hha*I revealed that the C-terminal half of the protein and the fully functional recognition site mediates sequence specific DNA recognition by such enzymes. Since the monospecific DNA MTase M.*Aqu*I is a heterodimer; therefore, in principle, this enzyme affords the opportunity to dissect out the determinants of DNA recognition among the monospecific DNA MTases. A comparison between the predicted amino acid sequences of the α and β subunits of M.*Aqu*I (Karreman and De Waard, 1990) and the sequence of the related enzyme M.*Hha*I (Cheng *et al.*, 1993), revealed that the α subunit constituted most of the AdoMet binding site, and contained the catalytic cysteine. The β polypeptide contained the TRD sequence together with homology blocks IX and X, including the C-terminal helix-loop-helix region. It is, therefore, an obvious candidate for sequence specific DNA recognition. In order to define whether the β subunit alone is able to discriminate between specific and nonspecific DNA sequences, we used purified Hisβ as well as both the specific and nonspecific oligonucleotides in order to obtain protein-DNA complexes.

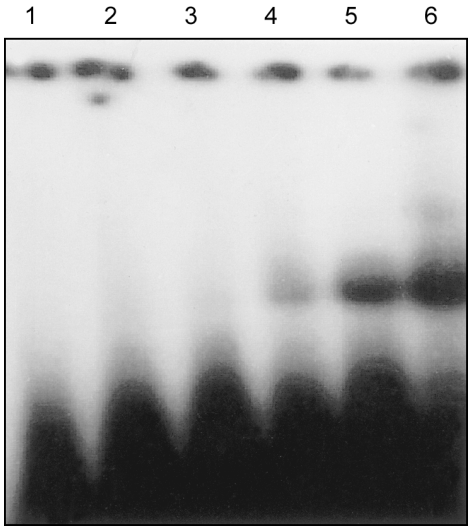

Fig. 2. Sequence specific DNA binding by the β subunit of M.*Aqu*I. Lanes 1-3: end-labeled nonspecific 30 mer duplex (NSD) DNA containing the sequence. ACCGGT in place of the M.*Aqu*I site, together with 0.5, 1 and 2 µM pure Hisβ, respectively, Lanes 4-6: end-labeled specific 30 mer duplex (SD) DNA containing a single recognition site (highlighted) for M.*Aqu*I and 0.5, 1 and 2 µM pure Hisβ, respectively.

SD: 5'GTACGAGCAGCT**CCCGGG**TCAGTCTGCCTA
5'TAGGCAGACTGA**CCCGGG**AGCTGCTCGTAC

SD and NSD were labeled at 5' ends with [γ-^{32}P] ATP using T4 polynucleotide kinase. The unincorporated label was removed by gel filtration using Sephadex G-25 resin and the spin column procedure. All of the reactions were carried out in a total volume of 20 µl, containing a final concentration of 1 nM labeled duplexes and a specified amount of Hisβ in a M.*Aqu*I binding buffer (20 mM Tris-HCl pH: 7.9, 100 mM NaCl, 1 mM EDTA, 7 mM β-mercaptoethanol). Each incubation was for 1 h at room temperature. Reaction mixtures were loaded on a 6% non-denaturing polyacrylamide (19 : 1) gel. The gel was run at 100V for 1 h in a TBE buffer, and dried on 3 MM Whatman paper under vacuum at 80°C for 45 min. The dried gel was exposed to X-ray film overnight at −70°C.

The β subunit binds to the oligonucleotide, which contains a specific recognition sequence for M.*Aqu*I. Increased binding of the β subunit to the specific DNA sequence was observed with increasing amounts of protein. Incubation of the β subunit with the nonspecific oligonucleotide did not produce a band shift (Fig. 2). In conclusion, our results clearly indicate that the β subunit alone contains all of the information for discrimination between nonspecific and specific DNA duplexes in the absence of the α subunit and cofactor. However, M.*Aqu*I requires both the α and β subunits for *in vitro* methylation of target DNA.

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