Novel Inhibitor of E. coli DNA Adenine Methyltransferase (Ecodam)

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Abstract—EcoDam is an adenine-N6 DNA methyltransferase that methylates the GATC sites in the Escherichia coli genome. DNA-adenine methylation is not present in higher eukaryotes including humans. These observations raise the possibility that dam inhibitors may be used as anti-microbial agents. Polyphosphate (Poly(P)) is an important metabolite and signaling molecule in prokaryotes and eukaryotes. Here, by using gel retardation experiments to investigate the competition of DNA binding by EcoDam in the presence of polyphosphate, we found that Poly (P) strongly interferes with DNA binding by EcoDam, while same concentration of monophosphate does not. In addition, we demonstrated that Poly (P) binding inhibits the activity of EcoDam and our results suggest that Poly (P) led to strong inhibition of the EcoDam catalytic activity, while monophosphate had only moderate effect.

Keywords—Antibacterial drugs, EcoDam inhibitors, Polyphosphate.

I. INTRODUCTION

The need for effective antibiotics targeting new microbial pathways is broadly appreciated, yet remains unfulfilled. Indeed, until the recent approval of an oxazolidinone, no new antibacterial drug had been approved for more than 25 years [1]. S-Adenosylmethionine (AdoMet)-dependent methylation is the most common form of post-replicative modification of DNA and represents one of the best understood epigenetic mechanisms [2]. This simple modification plays diverse roles in viruses, bacteria, plants and animals. In eukaryotes, DNA cytosine C5 methylation contributes to transcriptional regulation and genomic imprinting is essential for proper mammalian development, and its dysfunction is an early non-mutational path to tumorigenesis [3]. Bacterial DNA methyltransferases modify cytosines at C5 or N4 as well as adenine at N6 [4]. Most prokaryotic DNA MTases are parts of restriction/modification (RM) systems, which are widely distributed in the bacterial and archeal kingdoms. Orphan DNA MTases is a group of prokaryotic MTases that is not accompanied by a restriction enzyme. Two well-studied DNA methyltransferases are E.coli DNA adenine methyltransferase (EcoDam) enzyme that recognizes the GATC palindromic sequence and methylates adenine at N6-position and the CcrM (cell cycle-regulated DNA MTase) enzyme, originally identified in Caulobacter crescentus, which methylates the adenosine in the sequence 5’-GANTC-3’ (where “N” is any nucleotide) [5].

DNA adenine methyltransferase (Dam) has been reported to control the expression of a number of virulence genes [6,7]. In Salmonella, Haemophilus, and certain strains of Yersinia pseudotuberculosis, lack of Dam methylation causes attenuation of virulence in model animals [8,9]. Deletion of Dam erases DNA methylation pattern, which could alter the binding of regulatory proteins to a number of regions on the bacterial chromosome. In the absence of Dam, overexpression of genes could occur if GATC methylation blocked binding of an activator or enhanced the binding of a repressor. Conversely, under expression of a gene would occur in the absence of Dam if GATC methylation blocked binding of a repressor or enhanced binding of an activator. However, in both Yersinia pseudotuberculosis and Vibrio cholerae, virulence attenuation is observed if Dam methylase is overproduced [10, 11]. Albeit widespread, the involvement of Dam methylation in bacterial virulence is not universal; for instance, Dam’ mutants of Shigella flexneri are not attenuated [12].

Polyphosphate is a linear polymer of tens to hundreds of phosphate residues linked by high-energy phosphoanhydride bonds as in ATP. It has been found in all living organisms tested, ranging from bacteria to higher eukaryotes. In E. coli, the level of Poly (P) is very low in the exponential growth phase, but it increases up to 1000-fold in response to amino acid starvation and during the onset of the stationary phase. Poly(P) plays an important role as an energy source, as a regulator of gene expression, as a channel-forming component, and as a store of inorganic phosphate. Here we describe our initial success with an artificial Poly (P) as inhibitor of EcoDam.

II. METHODOLOGY

A. Expression and Purification of His-Tagged Dam

The protein was purified essentially as described before [13], by Ni-NTA affinity chromatography. E.coli strain HMS174 (DE3) transformed with the respective expression plasmid (pET28a-EcoDam) was grown overnight from a single colony at 37°C in 20 ml LB broth containing 25 µg/ml kanamycin. One liter of LB-Kan medium was inoculated with a 10 ml overnight culture, which was allowed to grow to an A600 of 0.8 at 37°C. After addition of 1 mM IPTG, cells were harvested by centrifugation at 2000 rpm, 4 °C in a JLA rotor for 20 minutes. The pellets were resuspended in 80 ml washing buffer (20 mM Hepes, 0.1 mM DTT, 500 mM NaCl, 10% glycerol, 20 mM imidazole, pH 8.0) and lysed by sonification (Branson sonifier,
5 cycles, where each cycle consists of 7 pulses of 15 sec followed by 1 min incubation time). Lysates were cleared by centrifugation for 1 hour at 20,000 rpm, 4 °C in a JA rotor and the supernatant applied to Ni-NTA column equilibrated with washing buffer. The column was washed twice with 60 ml washing buffer and eluted with 20 mM Hepes, 0.1 mM DTT, 500 mM NaCl, 10% glycerol, 200 mM imidazole, pH 8.0. The resulting protein preparation was extensively dialyzed first against 20 mM Hepes, 0.1 mM DTT, 1 mM EDTA, 300 mM NaCl, 10% glycerol, pH 7.5, and then against 0.1 mM DTT, 1 mM EDTA, 300 mM NaCl, 77% glycerol, pH 7.5; aliquots were stored at −20 °C. Protein concentrations were determined using the theoretical extinction coefficient ε280nm of 39.935 M⁻¹ cm⁻¹ for wild-type EcoDam [14].

**In vitro effect of Poly(P) on bacterial DNA methyltransferase (EcoDam)**

The effect of commercial Poly(P) (Sigma, order number S4379) with an average chain length of 45 orthophosphates was tested in the in vitro methylation assay using the purified bacterial DNA methyltransferase (EcoDam). DNA methylation activity of the complex was measured by the incorporation of titrated methyl groups from labelled S-methylation activity of the complex was measured by the bacterial DNA methyltransferase (EcoDam). DNA was tested in the in vitro methylation assay using the purified 39.935 M⁻¹ cm⁻¹ for wild-type EcoDam [14].

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**Electrophoretic Mobility Shift Assay (EMSA)**

To investigate the DNA binding by EcoDam in the presence of Poly (P), a 150-bp DNA fragment containing a single EcoDam target site (GATC) was used. The DNA substrate was amplified by PCR amplification from pET28a(+) using the following oligonucleotides: 5’-ATC CCA CTA CCG AGA TAT CCT CGG ACC-3’ and 5’-TGT CCG GTT TTC AAC AAA CCA TGC-3’ and radioactively labelled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). Gel retardation experiments were basically carried out as described [16]. As control, DNA binding studies were done after adding matching amounts of sodium monophosphate. As shown in Fig. 1 Poly (P) strongly interferes with DNA binding by EcoDam, while same concentration of monophosphate does not.

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**III. RESULTS AND DISCUSSION**

**A. DNA Binding of EcoDam by Poly(P)**

To investigate the competition of DNA binding by EcoDam in the presence of Poly (P), a 150-bp DNA fragment containing a single EcoDam target site (GATC) was used. The DNA substrate was amplified by PCR amplification from pET28a(+) using the following oligonucleotides: 5’-ATC CCA CTA CCG AGA TAT CCT CGG ACC-3’ and 5’-TGT CCG GTT TTC AAC AAA CCA TGC-3’ and radioactively labelled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). Gel retardation experiments were basically carried out as described [16]. As control, DNA binding studies were done after adding matching amounts of sodium monophosphate. As shown in Fig. 1 Poly (P) strongly interferes with DNA binding by EcoDam, while same concentration of monophosphate does not.
B. In Vitro Effect of Poly(P) on Bacterial DNA Methyltransferase (EcoDam)

In addition, we wanted to examine if Poly (P) binding inhibits the activity of EcoDam (Fig. 2). The effect of Poly (P) was tested in the in vitro methylation assay using the purified bacterial DNA methyltransferase (EcoDam). DNA methylation activity of the enzyme was measured by the incorporation of titrated methyl groups from labelled S-[methyl-3H] AdoMet (specific activity 2.6196 TBq/mmol, Perkin Elmer) into a biotinylated, hemi-methylated oligonucleotide substrate with a single GATC site using the avidin-biotin methylation kinetic assay as described [15]. As shown in Fig. 2 Poly (P) led to a strong inhibition of the EcoDam catalytic activity, while monophosphate (used as a control at matching concentrations) had only moderate effect.

The methylation reactions were initiated by the addition of DNA substrate. At 15, 30, 45, 60, 90, 120, 150, and 180 s, 2 µl aliquots of the reaction mixture were removed and quenched in 40 µl of unlabelled Ado Met.

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REFERENCES