



# Time-resolved fluorescence of 2-aminopurine in DNA duplexes in the presence of the EcoP15I Type III restriction–modification enzyme



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## ABSTRACT

EcoP15I is a Type III DNA restriction and modification enzyme of *Escherichia coli*. We show that it contains two modification (Mod) subunits for sequence-specific methylation of DNA and one copy of a restriction endonuclease (Res) subunit for cleavage of DNA containing unmethylated target sequences. Previously the Mod<sub>2</sub> dimer in the presence of cofactors was shown to use nucleotide flipping to gain access to the adenine base targeted for methylation (Reddy and Rao, J. Mol. Biol. 298 (2000) 597–610.). Surprisingly the Mod<sub>2</sub> enzyme also appeared to flip a second adenine in the target sequence, one which was not subject to methylation. We show using fluorescence lifetime measurements of the adenine analogue, 2-aminopurine, that only the methylatable adenine undergoes flipping by the complete Res<sub>1</sub>Mod<sub>2</sub> enzyme and that this occurs even in the absence of cofactors. We suggest that this is due to activation of the Mod<sub>2</sub> core by the Res subunit.

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## 1. Introduction

Since its introduction by Ward [1], 2-aminopurine (2AP) has proven to be a versatile probe of DNA and RNA structure and of the interaction of proteins with these polymers. This is because its fluorescence emission is intense, whereas the canonical bases are practically non-fluorescent, its excitation wavelength is longer than that of the canonical bases, enabling its selective excitation, and, most importantly, it has great sensitivity to its immediate environment [2–7]. 2AP fluorescence is normally highly quenched when it is stacked in a DNA double helix, particularly when electron transfer quenching mechanisms involving nearby guanine bases are active, but any structural perturbation can dramatically enhance its emission. This is particularly informative when using time-correlated single photon counting (TCSPC) to examine the fluorescence decay of 2AP, as demonstrated in previous studies of the nucleotide flipping mechanism used by DNA methyltransferases (MTases) [8–12], restriction endonucleases [13], DNA repair

**Abbreviations:** 2AP, 2-aminopurine; Mod, modification subunit; Res, restriction endonuclease subunit; TCSPC, time correlated single photon counting; RM, restriction and modification; MTase, methyltransferase; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl homocysteine.

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enzymes [14,15] and DNA polymerases [16,17]. TCSPC reveals a complex fluorescence decay curve typically requiring four distinct exponential decay processes for a full description [2–17]. These four decay components have been assigned to different structural fluctuations around the 2AP, including a fully base-stacked conformation and an extrahelical conformation with very short (<100 ps) and long (~10 ns) lifetimes, respectively [2–7]. In double-stranded DNA, the short lifetime component dominates the fluorescence decay. Flipping of the base induced by the enzyme greatly enhances the fractional amplitude of the long lifetime component at the expense of the short lifetime component and this change in amplitudes is a marker for base flipping.

The EcoP15I Type III restriction–modification (RM) enzyme comprises two copies of a Mod subunit, responsible for sequence recognition on the DNA and the modification of a specific adenine within the target sequence, and one or two copies of a Res subunit which cleaves DNA 25 base pairs downstream of the target sequence, 5'-CAGCAG-3', on the top strand and 27 base pairs downstream on the bottom strand [18–20]. Cleavage generally only occurs when a second target sequence, oriented in a head-to-head fashion, is present on the DNA and S-adenosyl methionine (SAM) and ATP are provided as cofactors. Cleavage is prevented by methylation of the second adenine in the recognition sequence. The dimer of Mod subunits can function as a MTase. The distance between the two sites can vary enormously [21] and the mechanism of site-to-site communication involves DNA looping, DNA

translocation or DNA sliding or some combination of these mechanisms [22–24].

The Mod subunit, responsible for site recognition and methylation, contains a typical SAM-dependent methylation domain [25] and base flipping of the target adenine into this catalytic domain would be anticipated [26]. Substitution of 2AP for the target adenine led, as expected, to an increase in 2AP emission when the Mod<sub>2</sub> dimer MTase and sinefungin, an analogue of SAM, was added [26]. However, it was also reported that 2AP, when substituted for the first adenine in the target sequence, a base which is not targeted for modification, also showed a fluorescence emission enhancement when the MTase and sinefungin were added. It was suggested that this 2AP was also subject to base flipping. However, since fluorescence emission intensity of 2AP is sensitive not only to base flipping but also to other DNA distortions [16,17] this interpretation is open to question.

In this paper we use TCSPC to examine the 2AP fluorescence decay from these two locations within the target sequence of EcoP15I. Rather than use the Mod<sub>2</sub> dimer MTase, we use the complete restriction endonuclease. The subunit composition of EcoP15I RM enzyme has been reported as Res<sub>2</sub>Mod<sub>2</sub> [27,28] and as Res<sub>1</sub>-Mod<sub>2</sub> [29,30]. We present evidence for base flipping at the position expected for the modification methylation reaction and for the absence of base flipping at other locations, whether within the target sequence or outside the target sequence. The subunit composition of the restriction enzyme was confirmed to be Res<sub>1</sub>Mod<sub>2</sub> in agreement with other measurements [29,30].

## 2. Materials and methods

### 2.1. EcoP15I Type III restriction endonuclease

EcoP15I (2500 units/mL, New England Biolabs, MA, USA) was used as supplied. EcoP15I consists of methylation (Mod) and restriction (Res) subunits. Each Res subunit (GenBank ID: CAG24073.1) is composed of 970 amino acid residues and each Mod subunit (GenBank ID: CAA29616.1) is composed of 645 amino acid residues. The molecular weights for the Res and Mod subunits are 110,957 Da and 74,222 Da respectively. ExPASy-ProtParam [31] (<http://web.expasy.org/cgi-bin/protparam/protparam>) was used to calculate extinction coefficient values of 81,600 M<sup>-1</sup> cm<sup>-1</sup> for Res and 81,710 M<sup>-1</sup> cm<sup>-1</sup> for Mod, assuming all cysteine residues were reduced.

### 2.2. Oligonucleotides

Duplexes 2AP1 and 2AP2 contained a 2AP base substituting the second and first adenine base within the EcoP15I recognition site (5′-CAGCAG-3′) respectively, Table 1. Duplex 2AP3 was a control sequence in which the 2AP base was placed outside the recognition site. The bases neighbouring the 2AP are the same in each oligonucleotide. To ensure that 2AP was present only in double stranded form, a 1.2:1 ratio of unlabelled to labelled DNA strands were

annealed by heating the two complementary strands at 90 °C for 5 min followed by cooling overnight. Extinction coefficients were calculated using Integrated DNA Technologies biophysics online software [32] (<http://biophysics.idtdna.com/>).

### 2.3. Size-exclusion chromatography

An analytical HPLC gel filtration column (30 cm × 0.46 cm Bio-sep-SEC-S3000, Phenomenonex) calibrated with protein standards (apoferritin 443 kDa, β-amylase 200 kDa, alcohol dehydrogenase 150 kDa, bovine serum albumen 66 kDa and carbonic anhydrase 29 kDa) was used to determine the molecular mass of EcoP15I in a buffer composed of 20 mM Tris-HCl, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 10 mM magnesium chloride, 7 mM β-mercaptoethanol, 200 mM sodium chloride, 0.1 mM EDTA, pH 6.5 at room temperature as previously described [33]. The low pH is required for the stability of the silica column material. The presence of salt in the buffer is required to prevent non-specific interaction between the protein and the column matrix. The flow rate was 0.5 mL/min and elution was monitored by fluorescence at 350 nm with excitation at 295 nm.

### 2.4. Densitometry of SDS-PAGE gels

Densitometry was performed of denaturing polyacrylamide gels using NIH ImageJ. Density calibration used the Kodak No. 3 calibrated step tablet with a density ranging from 0.05 to 3.05 OD. The absolute density value of the Res and Mod bands was calculated using (band density value)/(molecular mass of individual subunit).

### 2.5. Steady-state fluorescence measurements

Each duplex was buffered with 50 mM Tris-HCl, 20 mM sodium chloride, 10 mM magnesium chloride, pH 7.5. S-adenosyl-L-methionine (SAM, New England Biolabs) and S-adenosyl-L-homocysteine (SAH, Sigma-Aldrich) were used at 100 μM concentration when required. 200 μL samples of 500 nM duplex plus 2.5 μM EcoP15I (assuming a Res<sub>1</sub>Mod<sub>2</sub> stoichiometry) were incubated for 30 min at 25 °C prior to measurement. Steady-state fluorescence spectra were measured using a FluoroMax (Horiba Jobin Yvon) photon counting spectrofluorometer with a bandpass of 10 nm for both excitation (315 nm) and emission (370 nm) as previously described [34,35].

### 2.6. Time-resolved fluorescence measurements

Fluorescence decays were measured using the time-correlated single photon counting technique (TCSPC). The excitation source was the third harmonic of the pulse-picked output of a Ti:Sapphire femto-second laser system (Coherent, 10 W Verdi and Mira Ti:Sapphire), consisting of pulses of ~200 fs at 4.75 MHz repetition rate, at a wavelength of 315 nm. Fluorescence decays were measured in an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics. The instrument response of the system was ~80 ps FWHM. Fluorescence decay curves were analysed using a standard iterative reconvolution method based on the following equation:

$$I(t) = \sum_{i=1}^n A_i \exp\left(\frac{-t}{\tau_i}\right)$$

where  $A_i$  is the fractional amplitude and  $\tau_i$  is the fluorescence lifetime of the  $i$ th decay component. The value of  $A_i$  gives the fractional population of the fluorescent species with lifetime  $\tau_i$ . In order to determine the number of exponential terms required to fit the

**Table 1**

DNA duplexes used in this study. The underlined region of each duplex is the recognition site of EcoP15I. 2 represents the location of the 2AP base.

2AP1	(5′-3′)TAGGTCAGAATT <u>CAGC2G</u> ACCTTAAGTAGCC (3′-5′)ATCCAGTCTTAAG <u>TCGTC</u> TGGGATTTCATCGG
2AP2	(5′-3′)TAGGTCAGAATT <u>C2GCAG</u> ACCTTAAGTAGCC (3′-5′)ATCCAGTCTTAAG <u>TCGTC</u> TGGGATTTCATCGG
2AP3	(5′-3′)TAGGTC2GAATT <u>CAGCAG</u> ACCTTAAGTAGCC (3′-5′)ATCCAGTCTTAAG <u>TCGTC</u> TGGGATTTCATCGG

decay data, the quality of the fit was judged on the basis of the reduced chi-squared statistic,  $\chi^2$ , and the randomness of the residuals. Fluorescence decay curves were collected at three emission wavelengths (365, 380, 395 nm) with an excitation wavelength 315 nm and analysed globally (fitted simultaneously, with lifetimes,  $\tau_i$ , as common parameters) using Edinburgh Instruments FAST software. When measuring the duplexes in the absence of EcoP15I, the concentration of each duplex was 10  $\mu$ M. When measuring 2AP1 + EcoP15I, the concentration for 2AP1 was 0.5  $\mu$ M and the concentration of EcoP15I was 2.5  $\mu$ M in order to make sure DNA was fully bound. When measuring 2AP2 + EcoP15I and 2AP2 + EcoP15I, the duplexes and EcoP15I used at 10  $\mu$ M respectively to ensure full binding.

### 3. Results

#### 3.1. Size-exclusion chromatography and gel densitometry

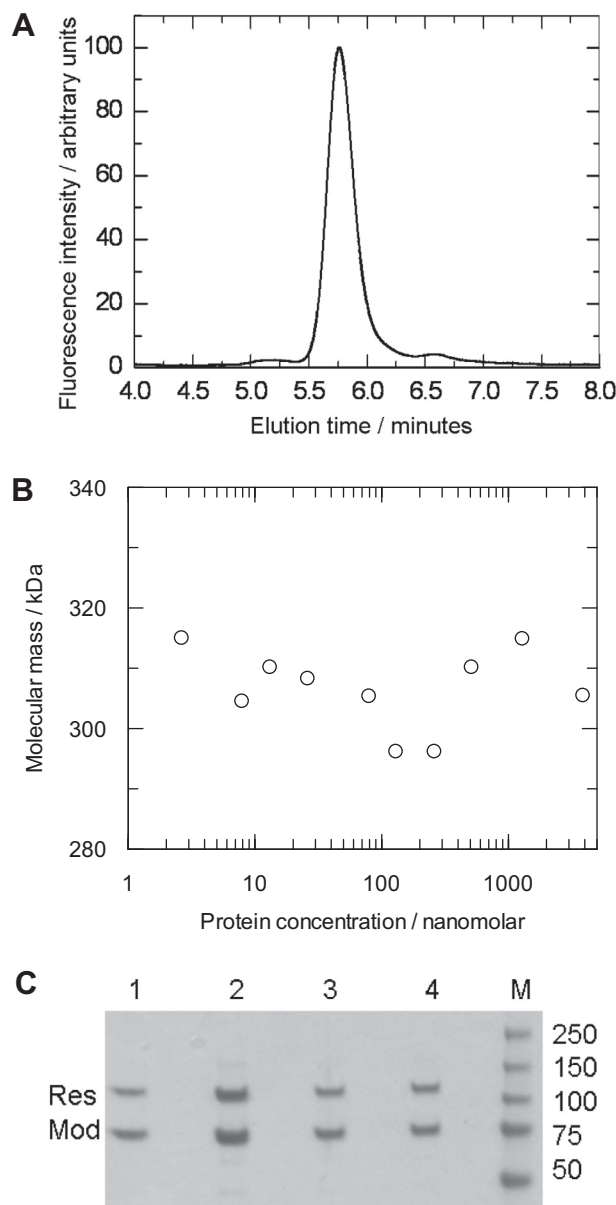
The subunit stoichiometry of EcoP15I has been reported as being either Res<sub>1</sub>Mod<sub>2</sub> or Res<sub>2</sub>Mod<sub>2</sub> [27–30]. This discrepancy will make the determination of protein concentration and the extent of DNA binding difficult so we first investigated the subunit composition of our sample of EcoP15I.

Enzyme samples with concentrations ranging from ~4000 nM to ~2 nM (estimated concentration of injected sample which will be diluted ~5-fold during the chromatography) were analysed by size exclusion chromatography. A single peak having a retention time of roughly 5.76 min, corresponding to a molecular mass of ~310 kDa, was observed for all sample concentrations, Fig. 1A. The apparent molecular mass versus concentration was constant indicating a stable quaternary structure in the concentration range employed, Fig. 1B. The average molecular mass differs from those calculated for Res<sub>1</sub>Mod<sub>2</sub> (260 kDa), Res<sub>2</sub>Mod<sub>1</sub> (296 kDa) and Res<sub>2</sub>Mod<sub>2</sub> (370 kDa). However, the values obtained from gel filtration assume a completely globular protein thus we can only conclude that the enzyme has a well-defined molecular mass.

To determine the subunit composition of the enzyme, gel densitometry was applied to samples run on denaturing polyacrylamide gels assuming a linear relationship between protein mass and density. The SDS-PAGE clearly demonstrated the high purity of EcoP15I, Fig. 1C, and showed two bands at molecular masses of ~110 kDa and ~75 kDa respectively, close to those expected for the Res and Mod subunits of EcoP15I. Densitometry (supplementary information) indicated that the molar ratio of Res and Mod subunits was 1:2.35, 1:1.84, 1:2.36 and 1:2.07 for the four different sample concentrations shown in Fig. 1C. Averaging these results indicated a ratio of 1 Res to 2.15  $\pm$  0.25 Mod and a Res<sub>1</sub>Mod<sub>2</sub> stoichiometry was assumed for the enzyme in all further experiments.

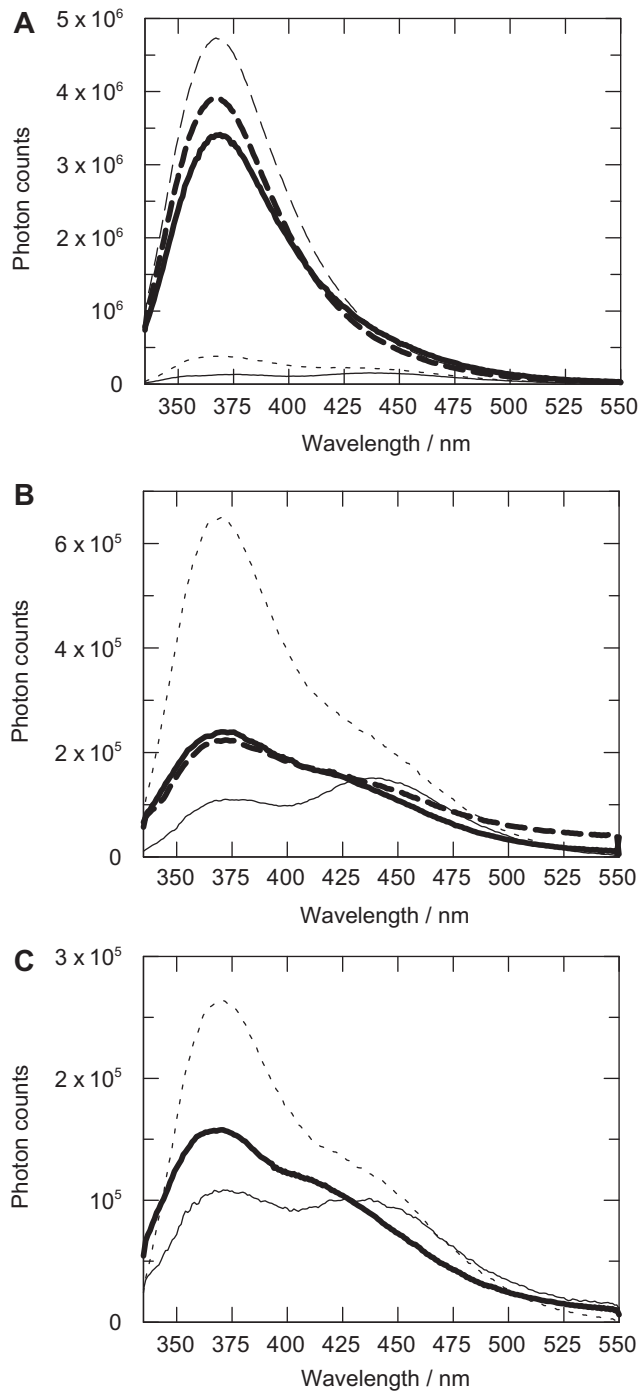
#### 3.2. Fluorescence spectroscopy

The emission spectra of the 2AP-containing DNA, Fig. 2, showed that the annealing of the complementary DNA strand to the strand containing 2AP caused a ~3 to ~5-fold reduction in peak intensity at ~375 nm. This quenching revealed the clear presence of a second emission peak at ~435 nm in the duplex DNA. This second peak has been attributed to fluorescence from a  $\pi$ - $\pi$  stacked dimer of 2AP and the adjacent base [5,7]. The fluorescence intensity of 2AP for all three duplexes at ~375 nm was very similar. The fluorescence decay parameters for the three duplexes all showed the characteristic four-exponential decay with lifetimes of ~50 ps, ~0.5 ns, ~3.0 ns and 10 ns and amplitudes of ~75%, ~10%, ~10% and ~5% respectively, Fig. 3 (supplementary information). These



**Fig. 1.** Size-exclusion chromatography and gel densitometry to investigate the quaternary structure of EcoP15I. (A) Typical elution profile (~1000 nM) showing a main single peak at ~5.76 min corresponding to an apparent molecular mass of 310 kDa. (B) Apparent molecular mass of the main elution peak plotted against the protein concentration of the injected solution. (C) SDS-PAGE analysis of EcoP15I enzyme after Coomassie Blue staining. The high purity of EcoP15I and the heavier staining of the mod subunit are evident. 0.375, 0.75, 0.4 and 0.5  $\mu$ L of the EcoP15I stock solution (34.3  $\mu$ M assuming Res<sub>1</sub>Mod<sub>2</sub>) were loaded in lanes 1–4 respectively. Lane M shows molecular mass markers as indicated.

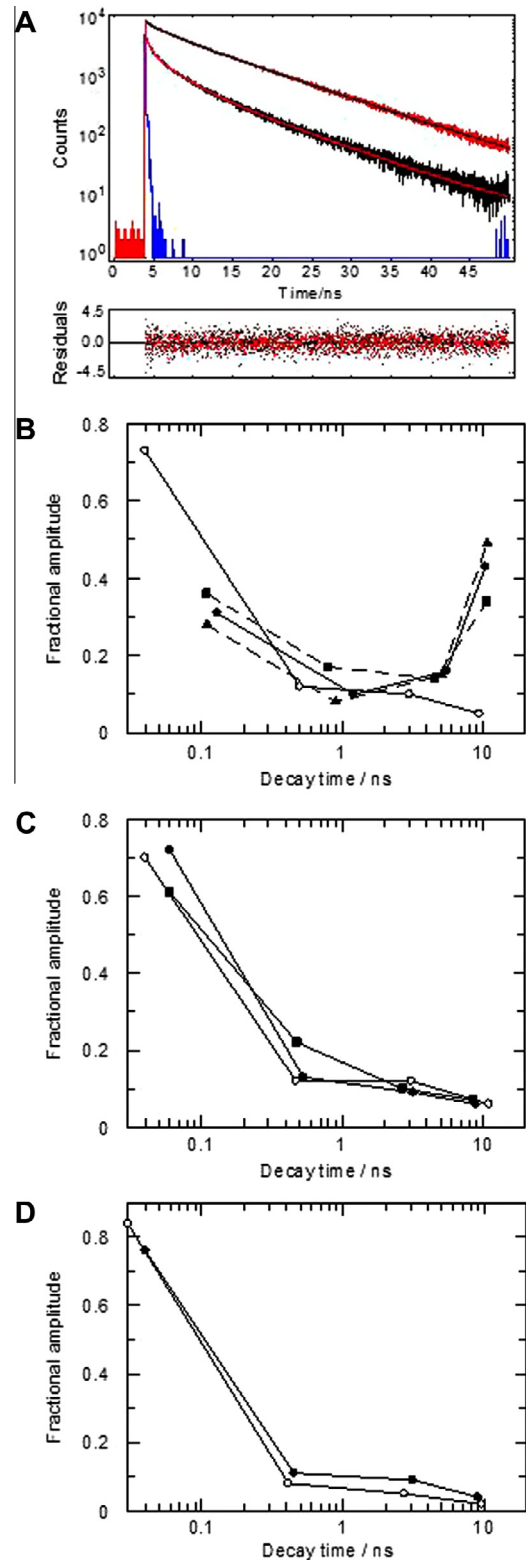
lifetimes and amplitudes all fall in the range of accepted values [2–17]. It is generally believed that the shortest lifetime corresponds to a tightly stacked, intrahelical base state where fast electron transfer quenching is dominant. The electron transfer quenching mechanism is most efficient when the adjacent base is a guanine, as used in these duplexes. The intermediate lifetimes are due to partially stacked conformations, while the longest lifetime is produced by the extrahelical state in which the 2AP base is unstacked. The highly stacked conformation accounts for over 70% of the emitting 2AP population. The unstacked state constitutes only ~5% of the population, due to the stability of



**Fig. 2.** Fluorescence emission spectra. (A) 2AP1 duplex (solid line, —), ss2AP1 (dotted line, ...), EcoP151 + 2AP1 (bold solid line, —), EcoP151 + 2AP1 + SAM (bold dashed line, — —), EcoP151 + 2AP1 + SAH (dashed line, - - -). (B) 2AP2 duplex (solid line, —), ss2AP2 (dotted line, ...), EcoP151 + 2AP2 (bold solid line, —), EcoP151 + 2AP2 + SAM (bold dashed line, — —). (C) 2AP3 duplex (solid line, —), ss2AP3 (dotted line, ...), EcoP151 + 2AP3 (bold solid line, —). Note changes in the magnitude of the ordinate axis.

Watson–Crick base pairing of the 2AP with thymine on the complementary strand.

The addition of EcoP151 to the duplexes showed a dramatic ~50-fold increase in intensity only for duplex 2AP1 which contains 2AP at the position targeted for methylation, Fig. 2B. This would be the scale of change expected for base flipping of the 2AP. The fluorescence decay showed a dramatic shift in the amplitudes of the four exponential components, with the longest decay component



**Fig. 3.** Fluorescence decay behaviour. (A) Fluorescence decays of duplex 2AP1 alone (black data) and 2AP1 + EcoP151 (red data) at an emission wavelength of 365 nm. The instrument response function is shown in blue. The data were fitted to a four component exponential decay (red line and black line respectively) and the residuals are shown in the panel below the decay curves. (B) Plots of fractional amplitude versus lifetime for 2AP1 duplex (○), duplex + EcoP151 (●), duplex + EcoP151 + SAM (■) and duplex + EcoP151 + SAH (▲). (C) Plots of fractional amplitude versus lifetime for 2AP2 duplex (○), duplex + EcoP151 (●), duplex + EcoP151 + SAM (■). (D) Plots of fractional amplitude versus lifetime for duplex 2AP3 (○) and duplex + EcoP151 (●). Standard deviations for all values of lifetimes and amplitudes were  $\pm 5\%$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

becoming dominant and again this is consistent with base flipping, Fig. 3B.

Duplexes 2AP2 and 2AP3 showed much smaller intensity increases of only 2-fold and 1.5-fold respectively upon the addition of the enzyme, Fig. 2B and C. Since both duplex 2AP2 and 2AP3 showed similar intensity changes due to the addition of EcoP15I, it would seem most probable that this is due to minor, non-specific distortion of the duplex, resulting in a small decrease in inter-base quenching, rather than base flipping in either of these duplexes. The absence of base flipping is strongly supported by the fluorescence decay data which showed almost identical decay parameters for both duplex 2AP2 and 2AP3 in the presence or absence of the enzyme, Fig. 3C and D.

The addition of SAM or the methylation product, S-adenosyl homocysteine (SAH), to the mixture of duplex 2AP1 and EcoP15I gave a further small fluorescence intensity increase of ~10% or ~30%, Fig. 2A. Fluorescence decay analysis indicated that this was due to slight increases in the magnitude and/or amplitude of the longest lifetime component, indicative of small changes in the population and local environment of the flipped 2AP. The addition of SAM to a mixture of EcoP15I and duplex 2AP2 had no effect on fluorescence intensity, Fig. 2B, or fluorescence decay, Fig. 3C.

#### 4. Conclusion

The clear conclusion from our work is that only the base at the methylation site, the second adenine in the target sequence of EcoP15I, is flipped by the Res<sub>1</sub>Mod<sub>2</sub> RM enzyme. The base flipping occurs whether SAM is present or not. The base located at the first adenine in the target sequence is not flipped, as the fluorescence decay behaviour of 2AP at this position is no different from that of 2AP located outside the target sequence; the decay parameters show that 2AP in both these positions remains almost entirely intrahelical and highly stacked on enzyme binding. This a further demonstration of the power of TCSPC to clarify data from fluorescence intensity experiments.

Previously, on the basis of fluorescence intensity measurements, Reddy and Rao [26] reported that 2AP at both of the adenine locations in the target would undergo flipping when the EcoP15I Mod<sub>2</sub> MTase and the cofactor analogue sinefungin were both added. Flipping, as indicated by the fluorescence enhancement, was not observed when only the MTase was added.

The apparent discrepancy between our results and those of Reddy and Rao [26] could be attributed to the use of the complete Res<sub>1</sub>Mod<sub>2</sub> enzyme rather than just the MTase. Perhaps the Res subunit enhances the interaction of EcoP15I with its DNA target in such a manner that the further addition of SAM or sinefungin is not required to induce base flipping by the bound enzyme? In other words the Res subunit activates the MTase for base flipping even if the cofactors are absent.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.04.162>.

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