Using the fluorescence decay of 2-aminopurine to investigate conformational change in the recognition sequence of the EcoRV DNA-(adenine-N6)-methyltransferase on enzyme binding

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HIGHLIGHTS

► The anomalous response of 2AP intensity to M.EcoRV binding is resolved by measuring the fluorescence decay.
► The fluorescence decay parameters of 2AP report unambiguously and informatively on DNA conformation and the DNA-enzyme interface.
► When M.EcoRV binds to its recognition sequence it induces different local distortions at the two adenine positions.
► The target 2AP is flipped, but its fluorescence is quenched by interaction with aromatic residues in the catalytic site.
► Bending of the duplex at the non-target site alleviates inter-base quenching and exposes the 2AP to solvent.

GRAPHICAL ABSTRACT
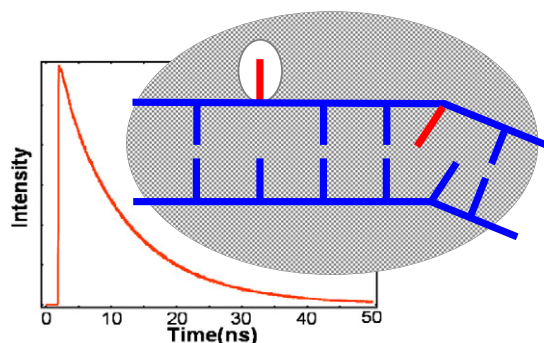
ABSTRACT

The EcoRV DNA methyltransferase methylates the first adenine in the GATATC recognition sequence. It is presumed that methylation proceeds by a nucleotide flipping mechanism but no crystal structure is available to confirm this. A popular solution-phase assay for nucleotide flipping employs the fluorescent adenine analogue, 2-aminopurine (2AP), substituted at the methylation target site; a substantial increase in fluorescence intensity on enzyme binding indicates flipping. However, this appeared to fail for M.EcoRV, since 2AP substituted for the non-target adenine in the recognition sequence showed a much greater intensity increase than 2AP at the target site. This anomaly is resolved by recording the fluorescence decay of 2AP which shows that the target 2AP is indeed flipped by the enzyme, but its fluorescence is quenched by interaction with aromatic residues in the catalytic site, whereas bending of the duplex at the non-target site alleviates inter-base quenching and exposes the 2AP to solvent.

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1. Introduction

DNA methyltransferases catalyse the methylation of DNA at a single base in a specific recognition sequence. This post-replicative modification adds information to the genome that is thought essential for DNA

transcription and replication, base-mismatch repair, mutagenesis, gene expression, genomic imprinting, gene silencing and X-chromosome inactivation [1]. On binding to a particular base sequence the enzyme isolates and extracts the target base into the active site through a mechanism termed nucleotide (or base) flipping, whereby the base is rotated around the sugar–phosphate backbone and brought into an extrahelical position so that it becomes accessible and DNA methyltransferases are able to chemically modify a part of DNA that is apparently inaccessible.

Base flipping is thought to be common to all DNA methyltransferases [2], but has only been proved in the few that have been successfully co-crystallised which include the adenine methyltransferases, M.TaqI [3], T4 Dam [4] and EcoDam [5]. The conserved catalytic motif for the adenine methyltransferases comprises D/N-P-P-Y/F/W. These structures show that flipped base stabilisation occurs through adenine-N6 hydrogen bonding with the D/N and P side chain oxygens and π-stacking with the Y/F/W aromatic ring.

Lack of available DNA–enzyme co-crystal structures stimulated the development of solution-based methods to determine the base flipping interaction. One particular strategy involves replacing the target base with a fluorescent analogue, 2-aminopurine (2AP) [6]. When intrahelical, 2AP is highly quenched through base stacking; its fluorescence intensity is very low. Destacking of 2AP results in an increase in fluorescence intensity, which can be taken as an indicator of base flipping. Since its first use to detect base flipping by M.EcoRI [6], this method has been applied to numerous MTases, most of which induce about a 10-fold increase in intensity of the 2AP target on DNA–enzyme binding [7–13]. However, in some cases, including M.EcoRV, the response of the steady-state fluorescence intensity of 2AP to enzyme binding has proved ambiguous, casting doubt on the reliability of 2AP as a probe of base flipping.

Although an increase in steady-state intensity is useful as an indicator of destacking of 2AP from the duplex, the magnitude of the intensity change is difficult to interpret, since different distributions of the 2AP population amongst the various conformational states of the duplex–enzyme complex may give similar steady-state fluorescence intensities. Time-resolved fluorescence spectroscopy gives a detailed picture of the environment around the 2AP fluorophore and the heterogeneity of the environment. 2AP in a homogeneous environment exhibits one lifetime; its fluorescence decay is monoexponential. The decay is multiexponential if the fluorophore is partitioned between several environments or conformations that provide distinctly different quenching efficiencies. Thus, 2AP-containing DNA duplexes typically show fluorescence decays described by four lifetime components, reflecting the existence of the duplex in a variety of conformational states. Changes in the fluorescence decay parameters on enzyme binding reveal the nature of the conformational perturbation in terms of the effect on the molecular environment of 2AP (reported by the fluorescence lifetimes) and the occupancy of the different conformations (reported by the amplitudes of the decay components).

Time-resolved fluorescence is becoming established as a powerful investigative tool for studying DNA–methyltransferase interaction because changes in the 2AP fluorescence decay on enzyme binding can be interpreted to give a detailed picture of the conformational distortion that is induced and an assessment of the extent of base flipping [14–16]. It has also been employed in studies on a DNA–polymerase [17] and on several DNA–endonucleases [18].

The EcoRV methyltransferase is one part of the EcoRV restriction modification system. The methyltransferase acts on the GATATC base sequence and methylates the first (5') adenine. On binding to this sequence, M.EcoRV contacts the major groove [19,20] and bends the DNA by about 60° [21]. Cross-linking studies with 5-iododeoxyuracil substituted for each adenine revealed a strong interaction between M.EcoRV and each adenine of GATATC, particularly the target adenine, and it is proposed that aromatic side chains intercalate the DNA helix, π-stack with the second adenine and cause the DNA to bend [22]. It

has been shown that M.EcoRV recognises the outer base pairs of the GATATC target site with residues corresponding to those that recognise the outer base pairs of the GATC target site in the related enzyme, EcoDam. Hence, M.EcoRV uses DNA bending to shorten the distance between the outermost base pairs of its recognition site, allowing it to be recognised as an expanded GATC site [23].

M.EcoRV methylates its substrate distributively so the cofactor must be bound before a specific DNA–enzyme complex can form [24] and the enzyme displays a burst of methylation activity. Fig. 1 shows the currently proposed binding model [24]. The enzyme binds its DNA substrate in two binding modes, specific (sp) and non-specific (nsp). The non-specific complexes form quickly and then slowly convert to specific complexes. It was proposed that the non-specific binding is predominantly electrostatic in nature, whereas the specific binding is predominantly hydrophobic, and that the specific complex is a base-flipped complex with the target base held inside a hydrophobic binding pocket [25]. However, no crystal structure is available and the base flipping mechanism of M.EcoRV still needs to be verified. During specific complex formation, contacts to the GAT part of the recognition sequence form first, then the ATC contacts form which mediate increased specificity over near-related GATC sites [22]. Kinetic studies reveal the presence of two separable specific complexes in slow equilibrium, ED_{sp} and ED'_{sp}[24].

In an attempt to use 2AP to reveal the base flipping mechanism in M.EcoRV [24], it was found that substituting 2AP for the non-target adenine in the recognition sequence produced an 11-fold intensity increase, whereas very little intensity change (1.4 fold increase) was found for 2AP at the (confirmed) target methylation site. This was surprising and contrary to the expectation that flipping of 2AP at the target site would result in a large intensity increase, whereas 2AP at the non-target site should be little affected. The sizeable increase in intensity for 2AP at the non-target site was suggested to be the effect of the known bending of the duplex by the enzyme and proved to be a useful indicator of specific binding in stopped flow studies, from which was deduced the binding model shown in Fig. 1. It was suggested that the absence of a definitive response of target 2AP was related to the hydrophobic nature of the binding pocket in which three aromatic residues appear to contribute to the binding of the flipped base.

We now report the use of time-resolved fluorescence of 2AP to reinvestigate the distortion of the duplex that occurs at each of the two adenine positions in the recognition sequence when M.EcoRV binds to DNA. 2AP has been substituted for adenine at each position, in turn, within the M.EcoRV recognition sequence, GATATC, in the unmethylated strand of the hemi-methylated duplex, and the fluorescence decays measured for the unbound duplexes and their complexes with M.EcoRV. The lifetime signatures obtained have allowed us to ascertain that specific binding of M.EcoRV to DNA distorts the duplex and destacks the base at both adenine positions in the recognition sequence, the nature of the destacked state is distinctly different at each position and the respective decay signatures are consistent with bending in the vicinity of the non-target base and flipping of the target base.
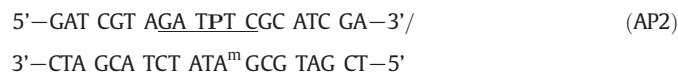
2. Materials and methods

2.1. Materials

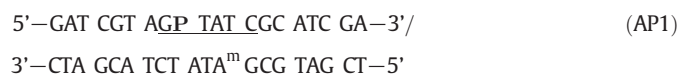M.EcoRV methyltransferase was purified as described previously [24]. Purified 20-mer oligonucleotides were purchased from Interactiva (Ulm),

$$E + D \rightleftharpoons ED_{nsp} \rightleftharpoons ED_{sp} \rightleftharpoons ED'_{sp}$$

Fig. 1. The M.EcoRV–DNA binding model [24], E represents M.EcoRV-AdoHcy, D represents DNA, nsp denotes a non-specific complex and sp denotes a specific complex.

PRIMM (Milan) or MWG(Erberberg). The following DNA substrates were used:

$$5'-\text{GAT CGT A}\underline{\text{G}\mathbf{P}\text{ TAT }}\text{CGC ATC GA}-3'/ \tag{AP1}$$

$$3'-\text{CTA GCA T}\underline{\text{CT ATA}}^{\text{m}}\text{GCG TAG CT}-5'$$

$$5'-\text{GAT CGT A}\underline{\text{GA TPT }}\text{CGC ATC GA}-3'/ \tag{AP2}$$

$$3'-\text{CTA GCA T}\underline{\text{CT ATA}}^{\text{m}}\text{GCG TAG CT}-5'$$

P is 2AP, A^m is N^6-methyl adenine and the underlined bases constitute the enzyme's recognition sequence. A methylated adenine base is used in the unlabelled strand to direct enzyme binding to the target base in the labelled strand.

Oligonucleotide strands were annealed using a 35% excess of the non-fluorescent strand to ensure that none of the 2AP-labelled strand was left unbound in the final solution. In order to ensure complete binding of the DNA by M.EcoRV, the enzyme complexes consisted of 2 μM DNA duplex, 6 μM M.EcoRV and 100 μM S-adenosyl-L-homocysteine (AdoHcy). (K_m, the Michaelis–Menten constant, for M.EcoRV with a 20-mer oligonucleotide and with S-adenosyl-L-methionine (AdoMet) is 0.30 μM and 12 μM respectively ±20% [25]) The buffer used was 50 mM NaCl, 50 mM HEPES, 1 mM EDTA and 0.001% Lubrol, pH 7.5. Measurements were performed on the free DNA and on the ternary enzyme complex. In the latter case, DNA was added to a pre-incubated mixture of the enzyme and cofactor.

2.2. Time-resolved fluorescence measurements

Fluorescence decays were measured using the time-correlated single photon counting technique (TCSPC), with a procedure that has previously been described [14,16]. The excitation source was the third harmonic of the pulse-picked output of a Ti-Sapphire femtosecond 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 318 nm. Fluorescence decays were measured in an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics. The instrument response of the system was ~70 ps FWHM. Fluorescence decay curves were analysed using a standard interative reconvolution method, assuming a multi-exponential decay function of the following form,

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

where A_i is the fractional amplitude and τ_i is the fluorescence lifetime of the ith decay component. The value of A_i gives the fractional population of the fluorescent species with lifetime τ_i. In order to determine the number of exponential terms required to fit the decay data, the quality of the fit was judged on the basis of the reduced chi-squared statistic, χ^2, and the randomness of the residuals, as described previously [16]. A typical decay curve (for duplex AP1), fitted multi-exponential function, residuals and instrument response function are shown in Fig. S1 of the Supplementary Information.

Decays were collected at several emission wavelengths (typically 370, 390 and 410 nm) and analysed globally using Edinburgh Instruments FAST software i.e. they were fitted simultaneously, with lifetimes, τ_i, as common parameters.

The fractional contribution of each decay component to the steady-state fluorescence intensity is given by the following equation.

$$F_i = \frac{A_i \tau_i}{\sum_i A_i \tau_i} \tag{2}$$

The fluorescence quantum yield of 2AP in DNA relative to that of free 2AP riboside, Φ_{rel}, can be calculated as follows,

$$\Phi_{\text{rel}} = \frac{\sum_i A_i \tau_i}{\tau_{\text{free}}} \tag{3}$$

where A_i and τ_i are the A factors and lifetimes derived from the fluorescence decay of 2AP in DNA and τ_{free} is the lifetime of 2AP riboside free in solution.

3. Results

3.1. Duplex AP2 and its ternary complex with M.EcoRV and AdoHcy

In duplex AP2, 2AP is substituted for the second (non-target) adenine in the recognition sequence. The fluorescence decay of 2AP in the free duplex requires three exponential terms to give a satisfactory fit. The observation of three decay times shows that 2AP exists in three distinct molecular environments that are distinguished by different quenching rates. The three decay times signify the existence of three conformational states of the duplex in which 2AP experiences different interbase interactions. The fluorescence lifetimes and their fractional amplitudes are given in Table 1. The value of the lifetime is indicative of the quenching efficiency (the shorter the lifetime, the more efficient the quenching) in a particular conformational state and fractional amplitude indicates the population of that conformation.

The shortest lifetime of 110 ps is characteristic of a highly stacked conformation in which 2AP is efficiently quenched by electron transfer from neighbouring bases (primarily guanine [26]). Almost half (46%) of the duplexes exist in this conformation. 53% of the duplexes display a lifetime of 400 ps, corresponding to a conformation in which 2AP is well stacked but is less susceptible to electron transfer from guanine. The conformation with the longest lifetime, 4.5 ns, has a population of only 1%. This lifetime is significantly shorter than that of 2AP riboside free in aqueous solution (10.6 ns) [14] indicating that the 2AP in this conformation is an intrahelical hydrophobic environment, but is imperfectly stacked.

The decay parameters of this duplex differ from those of numerous others that we [14–16,18,27,28] and others [29–32] have studied, which show 4-component decays, where the 4th component has a lifetime comparable to that of free 2AP and is characteristic of a conformation in which 2AP is extrahelical. Moreover, the other duplexes generally show a higher proportion of the lifetime (τ_3) with a value of a few nanoseconds. Thus, in the AP2 duplex, 2AP appears to be unusually tightly stacked. This is consistent with a previous study by Rai et al. [33] who found that 2AP inside the ATAT (and TATA) duplex sequence is highly constrained, immobile and inaccessible to acrylamide quenching.

As shown in Table 1 and illustrated graphically in Fig. 2, the fluorescence decay signature of the duplex changes dramatically on binding to M.EcoRV–AdoHcy. Four components are required to fit the

Table 1

Lifetimes (τ_i) and fractional amplitudes (A_i) for the unbound DNA substrates and their respective complexes with M.EcoRV–AdoHcy. The uncertainties in the values of lifetimes and A factors are: $\tau_1 \pm 10\%$; τ_2–$\tau_4 \pm 5\%$; A_1–$A_4 \pm 10\%$. The parameters in the presence of M.EcoRV are for a mixture of non-specific and specific complexes. Also reported are the calculated quantum yields relative to free 2AP riboside, Φ_{rel} (see Eq. (3)).

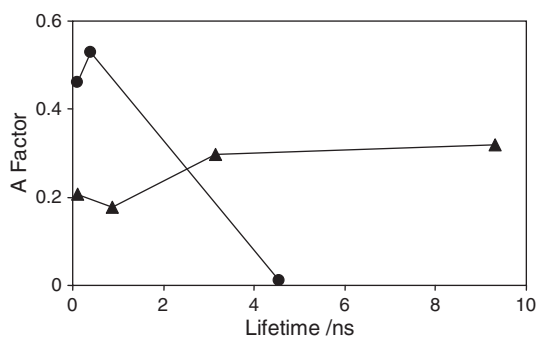| Solution Composition	τ_1/ ns	τ_2/ ns	τ_3/ ns	τ_4/ ns	A_1	A_2	A_3	A_4	Φ_{rel}
AP1	0.02	0.25	1.6	5.9	0.94	0.03	0.01	0.02	0.02
AP1/M.EcoRV/AdoHcy	0.09	0.62	2.9	7.6	0.45	0.23	0.19	0.13	0.16
AP2	0.11	0.40	4.5	–	0.46	0.53	0.01	–	0.03
AP2/M.EcoRV/AdoHcy	0.07	0.86	3.2	9.3	0.20	0.18	0.30	0.32	0.39

Fig. 2. Graphical representation of the decay parameters for the AP2 duplex when free in solution (circles) and in ternary complex with M.EcoRV and cofactor AdoHcy (triangles).

Fig. 3. Graphical representation of the decay parameters for the AP1 duplex when free in solution (circles) and in ternary complex with M.EcoRV and cofactor AdoHcy (triangles).

decay of the ternary complex. The additional long lifetime component of 9.3 ns (τ_4) is comparable to that of the free 2AP riboside in aqueous solution and indicates a sizeable population (32%) of a conformation in which 2AP is destacked and exposed to the solvent. The values of the other three decay times remain similar to those of the unbound duplex, but their fractional amplitudes have changed markedly. The population of conformations in which 2AP is efficiently quenched ($A_1 + A_2$) has decreased from 99% to 39%, and the conformational population (A_3) in which 2AP is only moderately quenched has increased from 1% to 30%. Thus, in the majority of the duplexes 2AP now experiences a weakly stacked or fully destacked environment. On the basis of the decay parameters, enzyme binding to the AP2 duplex is predicted to cause a 13-fold increase in relative quantum yield (and hence fluorescence intensity). This is in good agreement with the 11-fold increase in fluorescence intensity previously reported [24].

3.2. Duplex AP1 and its ternary complex with M.EcoRV and AdoHcy

As shown in Table 1, four exponential terms are required to describe the decay of unbound duplex AP1. However, almost all (94%) of duplexes exist in a conformation in which 2AP is extremely quenched, as a result of stacking with its immediate guanine neighbour, giving a lifetime of only 20 ps. The remaining duplexes show lifetimes corresponding to conformations with a range of less well stacked structures. As seen in AP2, 2AP in AP1 appears to be exceptionally firmly stacked in the duplex. The value of Φ_{rel}, 0.02, is significantly lower than that of AP2, reflecting the enhancement of quenching by direct stacking of 2AP with G.

Four components are also required to fit the decay of the ternary complex. Compared with the free duplex, all of the lifetimes have increased indicating a decrease in the extent of quenching of 2AP in all duplex conformations. As illustrated in Fig. 3, the A factors have changed significantly; the fraction of duplexes with 2AP in a highly stacked environment (A_1) has decreased from 94% to 45% and the fraction in weakly quenched or unquenched environments ($A_3 + A_4$) has increased from 3% to 32%. It is notable that the longest lifetime is characteristic of a hydrophobic rather than an aqueous environment. Thus, although 2AP has been destacked, it is not solvent-exposed but sees a hydrophobic environment where it experiences weak to moderate quenching interactions. From the Φ_{rel} values, an increase in fluorescence intensity of about 8-fold is predicted on M.EcoRV binding to this duplex. This is much greater than the 1.4-fold change reported before [24]. However, in the present study, fluorescence intensity measurements on the same sample stocks used for the lifetime measurements showed an increase in intensity of about a factor of 6. The difference in intensity response between the two studies can be attributed to the use of different cofactors, AdoMet in the previous work and AdoHcy in the present work, as discussed below.
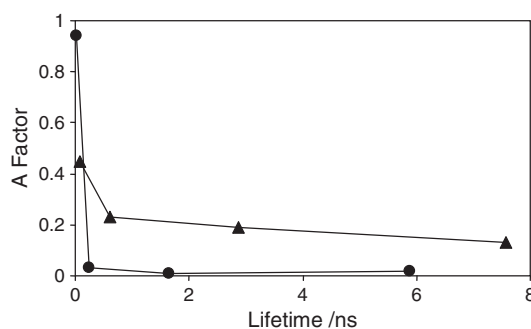
4. Discussion

When one compares the lifetime signature of each of the unbound duplexes with that of the corresponding enzyme complex (Figs. 2 and 3) it is evident that enzyme binding induces structural distortions at both adenine positions in the recognition sequence. Moreover, the different lifetime signatures of the two complexes indicate that the enzyme interacts differently with the target and non-target adenines, implying that the duplex is distorted differently at the two positions. We now consider the nature of these distortions.

4.1. Distortion of the duplex at the non-target adenine

The fluorescence decay of 2AP substituted at the non-target (second) adenine position in the GATATC recognition sequence (duplex AP2) indicates a major conformational distortion of the duplex on enzyme binding. Particularly notable is the appearance of a long lifetime component in the ternary complex which signifies the existence of 2AP in a destacked, solvent-exposed state, a conformation which is not seen in the free duplex. The observation of four decay parameters, all with sizeable populations, shows that the complex has a high degree of conformational heterogeneity.

To gain a more detailed picture of the conformational properties from the decay parameters we need to bear in mind the following points. (i) As discussed above and illustrated in Fig. 1, three enzyme–DNA complexes exist in equilibrium, a non-specific complex, ED_{nsp}, and two specific complexes ED_{sp} and ED'_{sp}[24].There is no change in fluorescence intensity of duplex AP2 on formation of ED_{nsp}, but there is an order of magnitude intensity increase on specific binding. (The two specific complexes have slightly different intensities, with ED'_{sp} lower by a factor of 0.7–0.9 than ED_{sp}.) The reported rate constants for interconversion of these complexes in the presence of AdoHcy indicate that the relative concentrations of the three complexes in the equilibrium mixture are: 23% ED_{nsp}, 34% ED_{sp}, 43% ED'_{sp} (with the caveat that the errors associated with the rate constants are high). (ii) In our experiments we observe the equilibrium mixture of the three complexes, each of which may exist in a range of conformational states. (iii) In a molecular system of this size and complexity, each lifetime does not, in fact, represent an individual conformation but represents a distribution of conformations with a range of (similar) lifetimes. Each fitted lifetime can be taken to represent the mean of a distribution of lifetimes.

The lifetime components τ_4 and τ_3, which are essentially absent in the unperturbed duplex, can be attributed to the combined population of specific complexes. (We cannot distinguish between ED_{sp} and ED'_{sp} in this experiment.) Thus, in the specific complexes, base-stacking of 2AP is greatly disrupted and 2AP has a high propensity to exist in a destacked, solvent-exposed state. These observations are entirely consistent with bending of the DNA backbone on specific complex

formation, with the non-target adenine positioned at the apex of the bend, as proposed previously [21,23,24]. The persistence of a significant fraction (20%) of a highly stacked conformation (τ_1) is consistent with the presence of ED_{nsp} in the equilibrium mixture. It is likely that both τ_1 and τ_2 are associated predominantly with ED_{nsp}, but we cannot rule out the possibility that there may be a contribution to these decay components from conformations of ED_{sp}/ED'_{sp} in which 2AP samples a well-stacked intrahelical environment. From the A factors, we can therefore estimate that specific complexes, ED_{sp}/ED'_{sp} together, constitute at least 62% ($A_3 + A_4$) of the equilibrium population and the non-specific complex, ED_{nsp} constitutes no more than 38% ($A_1 + A_2$). These estimates are consistent with those estimated from the kinetic model, 77% specific, 23% non-specific, noted above [24]. Therefore, we can infer that the decay of 2AP in the specific complex of duplex AP2 consists predominantly of 2 lifetime components (τ_3 and τ_4) with approximately equal amplitudes, as indicated in Table 2.

4.2. Distortion of the duplex at the target adenine

Consider first the partitioning of the population between specific and non-specific complexes. Lifetime components τ_2, τ_3, and τ_4 (Table 1) can be attributed to ED_{sp}/ED'_{sp}, given that the populations of these in the free duplex were negligibly small. The shortest lifetime component, τ_1, is clearly attributable to ED_{nsp}, but the population (45%) of highly quenched conformations is too high to be due to ED_{nsp} alone (vide supra). Thus, about 15% of 2AP exists in highly quenched conformations in ED_{sp}/ED'_{sp}. Using these arguments, we can infer that the decay of 2AP in the specific complex of duplex AP1 consists of 4 lifetime components, with τ_1 having an amplitude of 0.15 (A_1 in Table 2), and τ_2, τ_3, and τ_4 having a combined amplitude of 0.85, giving the re-normalised values of A_2 to A_4 shown in Table 2.

Previously, we have established the decay parameters for 2AP flipped into the active site of another adenine-N6-methyltransferase, M.TaqI, from time-resolved fluorescence measurements on a crystalline complex of known structure [15]. These parameters, together with those of the same 2AP-flipped complex in solution are given in Table 3. The catalytic motif of M.TaqI is NPPY and the flipped 2AP fluorescence is quenched by π-stacking with the tyrosine residue in this motif, giving rise to the 0.93 ns and 2.3 ns decay times of the crystalline complex. The latter decay components together account for 97% of the 2AP population, the remaining 3% has a lifetime of 8.6 ns, indicative of exposure of 2AP to an unquenched hydrophobic environment (the population of the latter conformation is too small for it to be seen in the X-ray structure). The lifetime signature is essentially conserved in the M.TaqI solution-phase complex; the slightly shorter decay times suggest greater conformational mobility of 2AP, allowing access to more efficiently quenched conformations.

The values of τ_2, τ_3 of the AP1 specific complex with M.EcoRV (Table 2) are very similar to those of the base-flipped M.TaqI complex in solution, as would be expected if they were due to 2AP flipped from the duplex and resident in the M.EcoRV active site, in the vicinity of the DPPY catalytic motif. As in M.TaqI, the majority of the flipped 2AP population (65%) exists in these quenched conformations in the M.EcoRV active site. The fraction of 2AP in an unquenched, hydrophobic environment (τ_4) is somewhat higher in M.EcoRV than in M.TaqI (20% compared with 8%) and in M.EcoRV there is also a significant

population (15%) of highly quenched 2AP ($\tau_1 = 90$ ps). It is evident that the AP1 specific complex is conformationally heterogeneous, the flipped 2AP is mobile and shows no strong preference for a particular orientation in the catalytic pocket. The very short, 90 ps, decay time may indicate that the flipped base makes excursions back into the stacked interior of the duplex or may be due to a strong interaction with an aromatic residue in the catalytic pocket. We note that there is evidence of an additional tyrosine and a tryptophan near the adenine binding site which appear to contribute to the binding of the flipped base [34–36]. Recently, we have reported similar very short decay components for 2AP flipped into the binding pockets of restriction enzymes Ecl18kI (70 ps) and EcoRII-C (80 ps), where crystallography shows that the flipped base interacts closely with tryptophan and tyrosine, respectively [18].

Interaction of the flipped base with tyrosine in the M.EcoRV active site is also implied by studies of related methyltransferases, T4 Dam and EcoDam. It has been suggested that the GAT-interacting module in M.EcoRV is similar to that in T4 Dam and EcoDam [37]. Co-crystal structures of these two Dam enzymes with their DNA substrates are available. Both structures show a flipped adenine in the vicinity of the DPPY motif [4,5]. Additionally, the T4 Dam structure shows a non-catalytic conformation in which the flipped target adenine is stabilised on the surface of the enzyme [4]. A similar conformation, where the 2AP is flipped, but not strongly quenched by the enzyme, may be responsible for the 7.6-ns lifetime in the AP1-M.EcoRV specific complex.

We return now to the effect of the cofactor, AdoMet/AdoHcy, on the response of the fluorescence intensity of 2AP to enzyme binding. When 2AP is at the non-target position (duplex AP2) the increase in intensity appears independent of the identity of the cofactor: essentially the same intensity increase was seen in the present study, using AdoHcy, and in the previous study [24], using AdoMet. In contrast, when 2AP is at the target position (duplex AP1) a substantially greater intensity increase was seen with AdoHcy as the cofactor than with AdoMet. These observations support our interpretation of the decay parameters. In the ternary complex, the non-target 2AP remains remote from the active site and is not influenced by the cofactor, whereas the target 2AP is flipped into the active site and is sensitive to the nature of the cofactor. The presence of AdoMet increases the quenching of the flipped 2AP fluorescence, implying that a greater proportion of the flipped 2AP population interacts with aromatic residues in the catalytic pocket. It seems that replacing AdoHcy by AdoMet results in a transfer of population from the unquenched or moderately quenched, flipped conformations (τ_3 and τ_4) to the more highly quenched, flipped conformation (τ_2). On the basis of Eq. (2), it is evident that almost all (98%) of the steady state fluorescence intensity of the equilibrium mixture of the AP1/M.EcoRV/AdoHcy complexes arises from the specific complexes and, in turn, the vast majority (91%) of the intensity of the specific complexes arises from lifetime components τ_3 and τ_4. If the total population of the latter two components were to be transferred to τ_2, this would result in a decrease in the fluorescence intensity by a factor of 4.5, close to what we observe when AdoHcy is replaced by AdoMet.

Influence of the cofactor on the conformation of the base-flipped complex has been observed previously for the related methyltransferases, EcoDam and T4Dam. Stopped flow studies of EcoDam, monitoring 2AP fluorescence intensity, indicated that although base

Table 2
Decay parameters inferred for duplexes AP1 and AP2 in their respective specific complexes with M.EcoRV–AdoHcy.

Specific Complex	τ_1/ ns	τ_2/ ns	τ_3/ ns	τ_4/ ns	A_1	A_2	A_3	A_4
AP1/ M.EcoRV/AdoHcy	0.09	0.62	2.9	7.6	0.15	0.35	0.3	0.2
AP2/ M.EcoRV/AdoHcy	–	–	3.2	9.3	–	–	0.5	0.5

Table 3
Decay parameters for 2AP flipped into the active site of M.TaqI in the crystalline state and in solution [15]. AETA is the cofactor analogue 5′-(2-(amino)ethylthio]-5′-deoxyadenosine. The uncertainties in the values of lifetimes and A factors are: τ_2–$\tau_4 \pm 5\%$; A_2–$A_4 \pm 10\%$.

2AP-flipped Complex	τ_1/ ns	τ_2/ ns	τ_3/ ns	τ_4/ ns	A_1	A_2	A_3	A_4
DNA/M.TaqI/AETA crystal	–	0.93	2.3	8.6	–	0.46	0.51	0.03
DNA/M.TaqI/AETA soln	–	0.55	1.9	7.3	–	0.54	0.38	0.08

flipping occurs in the presence of AdoHcy, binding of the target base in the active site (*via* stacking with the conserved tyrosine residue) is enhanced by AdoMet [9]. Moreover, the co-crystal structure of T4Dam–DNA with AdoHcy shows that the target adenine is flipped out but not fully ordered in the active site, whereas the structure with the AdoMet analogue, sinefungin, shows the flipped adenine in close contact with the conserved DPPY catalytic motif [4].

5. Conclusion

The fluorescence decay of 2AP clearly reveals that when M.EcoRV binds to its recognition sequence it induces different local distortions at the two adenine positions. The decay parameters of 2AP substituted at the target site are indicative of flipping of the base into the hydrophobic environment of the catalytic site, where its fluorescence is quenched by interaction with tyrosine in the DPPY motif, and possibly with other aromatic residues. The decay signature closely resembles that seen previously for other base-flipped complexes where there is crystallographic evidence for such quenching interactions in the active site.

When 2AP is substituted for adenine at the non-target position, its fluorescence decay reports that specific binding causes destacking of the base and its exposure to the solvent environment. This supports the previous proposal of duplex bending, with the non-target adenine at the apex of the bend.

The information on the conformational population that can be gleaned from the decay parameters supports previous evidence for specific and non-specific binding. Moreover, the partitioning of the duplex population between non-specific and specific complexes that can be inferred is consistent with that deduced from previously reported binding kinetics.

The decay parameters predict that enzyme binding causes a greater increase in fluorescence intensity for 2AP at the non-target site, as observed in steady-state measurements. This is the consequence of destacking of the non-target base from the quenched duplex interior into a relatively unquenched solvent–exposed state, whereas the target base is transferred into the active site where it encounters quenching interactions.

The M.EcoRV–DNA system has become well known as an example of the failure of 2AP fluorescence intensity to reveal nucleotide flipping. This study illustrates how the measurement of time-resolved fluorescence can enhance the utility of 2AP, enabling it to report unambiguously and informatively on DNA conformation and the DNA-enzyme interface.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2011.09.001.

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