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2-Aminopurine Flipped into the Active Site of the Adenine-Specific DNA Methyltransferase M.TaqI: Crystal Structures and Time-Resolved Fluorescence

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Abstract: We report the crystal structure of the DNA adenine-N6 methyltransferase, M.TaqI, complexed with DNA, showing the fluorescent adenine analog, 2-aminopurine, flipped out of the DNA helix and occupying virtually the same position in the active site as the natural target adenine. Time-resolved fluorescence spectroscopy of the crystalline complex faithfully reports this state: base flipping is accompanied by the loss of the very short (~50 ps) lifetime component associated with fully base-stacked 2-aminopurine in DNA, and 2-aminopurine is subject to considerable quenching by π-stacking interactions with Tyr108 in the catalytic motif IV (NPPY). This proves 2-aminopurine to be an excellent probe for studying base flipping by M.TaqI and suggests similar quenching in the active sites of DNA and RNA adenine-N6 as well as DNA cytosine-N4 methyltransferases sharing the conserved motif IV. In solution, the same distinctive fluorescence response confirms complete destacking from DNA and is also observed when the proposed key residue for base flipping by M.TaqI, the target base partner thymine, is substituted by an abasic site analog. The corresponding cocrystal structure shows 2-aminopurine in the active site of M.TaqI, demonstrating that the partner thymine is not essential for base flipping. However, in this structure, a shift of the 3′ neighbor of the target base into the vacancy left after base flipping is observed, apparently replicating a stabilizing role of the missing partner thymine. Time-resolved fluorescence and acrylamide quenching measurements of M.TaqI complexes in solution provide evidence for an alternative binding site for the extra-helical target base within M.TaqI and suggest that the partner thymine assists in delivering the target base into the active site.

1. Introduction

DNA methyltransferases (MTases) catalyze the transfer of the activated methyl group from the ubiquitous cofactor *S*-adenosyl-L-methionine (AdoMet) to the target base within their recognition sequences. DNA MTases are divided into three groups, depending on their chemo- (adenine or cytosine) and regio- (N4 or C5 of cytosine) selectivity: DNA adenine-N6, DNA cytosine-N4 and DNA cytosine-C5 MTases. All classes occur in prokaryotes, e.g. as part of restriction-modification systems, but in eukaryotes only DNA cytosine-C5 MTases are found, where they play an important role in cell differentiation, transcriptional regulation of gene expression (gene silencing), genomic imprinting, and carcinogenesis. (See refs 1−11 for recent comprehensive reviews).

DNA MTases as well as many DNA glycosylases gain access to their target 2′-deoxyribonucleotide by base flipping (also called nucleotide flipping).[3,11] The target nucleotides, which are inaccessible when buried within canonical B-DNA, are rotated out of the DNA helix and placed into the active sites of the enzymes where catalysis takes place. Base flipping was originally demonstrated by X-ray crystal structure analysis of the protein-DNA complex of the DNA cytosine-C5 MTase

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M.HhaI.[12] More recently, flipping of adenine was demonstrated for the DNA adenine-N6 MTases M.TaqI,[13] T4 Dam,[14] and *Escherichia coli* Dam.[15] M.TaqI methylates the target adenine within the double-stranded DNA sequence 5′-TCGA-3′. In the X-ray structure of the ternary complex between M.TaqI, DNA, and the cofactor analog 5′-[2-(amino)ethylthio]-5′-deoxyadenosine (AETA), a closure of the two protein domains was observed with respect to the binary complex of M.TaqI with its cofactor or analogs.[16] Thus, the DNA bound between the two domains is compressed at the target base pair position and the target base partner thymine is shifted into the hole left by the flipped adenine, where it π-stacks with the 5′ neighbor guanine of the extrahelical target adenine. In this interstrand-stacked position, the partner thymine was interpreted to stabilize the apparent abasic site formed after target base flipping, as demonstrated in binding studies with M.TaqI and duplexes containing polycyclic aromatic DNA-base surrogates at the partner position.[17] Furthermore, a push mechanism was proposed in which M.TaqI—by domain closure—pushes actively against the target base partner thymine, which in turn displaces the target adenine from the inside of the DNA helix.[18]

However, it is not always possible to obtain evidence of base flipping by using X-ray crystallography, as crystals are not always obtainable. Therefore, spectroscopic methods have often been adopted. 2-Aminopurine (**2Ap**) is a fluorescent analog of adenine (6-aminopurine) and can form two Watson−Crick-like hydrogen bonds with thymine in DNA. Duplexes containing **2Ap**-T base pairs instead of A-T base pairs have a very similar conformation and a slightly reduced thermal stability.[19−22] The fluorescence properties of **2Ap** respond to its molecular environment. Compared to the free fluorophore or **2Ap**-ribonucleoside in aqueous solution, its fluorescence intensity is drastically quenched when incorporated in DNA due to base stacking interactions.[23] In this position, it is buried inside the DNA and almost inaccessible to the solvent, as determined by acrylamide quenching experiments.[24] However, when its base partner is missing and **2Ap** faces an abasic site analog, the fluorescence intensity and solvent accessibility rise due to an increased extra-helical population of **2Ap**.[24−26]

The change in steady-state **2Ap** fluorescence intensity caused by destacking of **2Ap** in labeled DNA has been used to detect and characterize base flipping by a range of DNA MTases.[27−39] In almost all studies, substitution of the target base by **2Ap** gave the greatest increase in fluorescence intensity upon addition of enzyme. This is also true for the M.EcoP15I MTase (in the presence of Sinefungin), but replacement of a nontarget adenine by **2Ap** within the recognition sequence also led to a comparable increase in fluorescence intensity.[40] Furthermore, for the M.EcoRV MTase, the opposite was observed. **2Ap**-substitution of another adenine within the recognition sequence, rather than the target adenine, gave the only detectable change in fluorescence intensity upon binding of the enzyme.[41,42] Thus, conformational changes other than base flipping can lead to substantial changes in **2Ap** fluorescence intensity. A change in steady-state fluorescence intensity necessarily indicates changes in the environment of a fluorophore but the opposite is not necessarily true. Changes in the environment of a fluorophore may not be apparent in the steady-state fluorescence intensity.

However, time-resolved fluorescence spectroscopy gives a more detailed picture of the environment around a fluorophore. A fluorophore 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. A shorter lifetime indicates that the fluorophore is more efficiently quenched. As an example, free **2Ap** (2′-deoxy)ribonucleoside in aqueous solution possesses a high quantum yield and a lifetime of $\sim$10 ns.[43−46] It has been shown that **2Ap**, when incorporated in DNA, exists in (three to) four distinguishable states of which the predominant one is the intra-helical, π-stacked, highly quenched (low quantum yield) state with a lifetime of tens of picoseconds.[20,25,47,48]

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Table 1. Abbreviations and Sequences of DNA Duplexes[a]

Abbreviation	Duplexes used in crystals	Abbreviation	Duplexes used in solution
10-A/T	1 2 3 4 5 6 7 8 9 10 5'-GT TCGA TGTC-3' 3'-CA **M**GCT ACAG-5' 20 19 18 17 16 15 14 13 12 11	14-A/T	5'-GCCGC TCGA TGCCG-3' 3'-CGGCG **M**GCT ACGGC-5'
10-**2Ap**/T	5'-GT TCG**2Ap** TGTC-3' 3'-CA **M**GCT ACAG-5'	14-**2Ap**/T	5'-GCCGC TCG**2Ap** TGCCG-3' 3'-CGGCG **M**GCT ACGGC-5'
10-**2Ap/D**	5'-GT TCG**2Ap** TGTC-3' 3'-CA **M**GC**D** ACAG-5'	14-**2Ap/D**	5'-GCCGC TCG**2Ap** TGCCG-3' 3'-CGGCG **M**GC**D** ACGGC-5'

[a] **M** represents N6-methyladenine, **2Ap** 2-aminopurine, and **D** a 1,2-dideoxy-D-ribose abasic site analog. The palindromic 5′-TCGA-3′ recognition sequence of M.TaqI (separated by space from the rest of the sequence) contains two symmetry related target bases, and by placing **M** at one target position (hemimethylated duplex), M.TaqI binding is oriented for flipping the other target base. For 10-A/T, numbering is included and the target adenine (A6) and its base partner thymine (T15) are highlighted. The 3′ neighbor thymine (T7) and the 5′ neighbor guanine (G5) of the target base, which π-stack in complex B of the 10-**2Ap**/**D**·M.TaqI·AETA crystal structure (T7 shift), are underlined.

Recently, Neely et al. used time-resolved fluorescence spectroscopy to investigate the decays of **2Ap** fluorescence of several **2Ap**-labeled DNA duplexes alone and when bound to the DNA cytosine-C5 MTase M.HhaI or its T250G[28] variant.[48] They reported a dramatic decrease of the fractional amplitude of the shortest lifetime assigned to the intra-helical state in DNA upon binding of M.HhaI T250G when **2Ap** was at the target position. In a single crystal containing **2Ap**-labeled DNA complexed with M.HhaI T250G and *S*-adenosyl-L-homocysteine (AdoHcy, the cofactor product formed after methyl group transfer), the lifetime corresponding to the intra-helical state of **2Ap** was completely absent and the crystal structure shows that the **2Ap** was extra-helical. Hence, this destacking fluorescence response constitutes an unambiguous signature of base flipping. However, the crystal structure shows that **2Ap** is not located in the active site but binding occurred in a remote position.

Here, we report the crystal structures and time-resolved fluorescence spectroscopy of ternary complexes between M.TaqI, the cofactor analog AETA and two different **2Ap**-substituted DNA duplexes. One duplex, in which **2Ap** is paired with thymine, mimics the natural substrate, whereas in the second duplex, thymine is replaced by a stable abasic site analog to elucidate the role of the partner thymine in base flipping. In parallel with these studies of **2Ap** in structurally defined, crystalline environments, complementary time-resolved fluorescence measurements were made on the free DNA duplexes and their binary and ternary M.TaqI complexes in solution. Acrylamide quenching experiments of the steady-state fluorescence of **2Ap** were performed to further characterize the solvent accessibility of **2Ap** in the two binary solution complexes.

2. Materials and Methods

Materials. Synthesis and purification of oligodeoxynucleotides (ODN) is detailed in the Supporting Information. Duplexes (see Table 1 for sequences) were formed by heating complementary ODN in binding buffer (20 mM Tris/HOAc, 10 mM $Mg(OAc)_2$, 50 mM KOAc, 1 mM DTT, 0.01% Triton X-100, pH 7.9) without Triton X-100 (at least 10 μM) or crystallization buffer (*vide infra*) to 95 °C and slow cooling to room temperature within 2−3 h. For the solution measurements, a 50% excess of the nonfluorescent ODN was used to ensure complete annealing of the **2Ap**-labeled ODN.

M.TaqI was overexpressed and purified as described before.[13,27] Concentration of M.TaqI was estimated by the method of Bradford[49] or by UV absorption at 280 nm using an extinction coefficient of 86 600 L mol^{-1} cm^{-1}. 5′-[2-(Amino)ethylthio]-5′-deoxyadenosine (AETA) was synthesized from 5′-chloro-5′-deoxyadenosine by nucleophilic substitution with cysteamine.

Crystallization. Crystallization of ternary M.TaqI complexes for X-ray crystallography and time-resolved fluorescence measurements was achieved as described for M.TaqI in complex with 10-A/T and AETA[13] with some modifications detailled in the Supporting Information.

Data Collection and Structure Determination. Cocrystals with dimensions of 10 × 10 × 100 to 10 × 30 × 100 μm^3 were harvested with a nylon fiber loop from a crystallization droplet, transferred quickly into a cryo protection solution (12% PEG6000 for 10-**2Ap**/T·M.TaqI·AETA or 12% PEG8000 for 10-**2Ap**/**D**·M.TaqI·AETA, 17.1% glycerol, 200 mM NaCl, 100 mM Tris/HCl, pH 7.3), and frozen in liquid nitrogen. Loops containing cocrystals were placed onto goniometer heads, and X-ray diffraction data were collected using a synchrotron beam source (European Synchrotron Radiation Facility, ESRF, Grenoble, France, see Table S1 of the Supporting Information for details on beamlines and detectors) under a nitrogen stream at 100 K.

Data processing was performed using the programs XDS/XSCALE[50] and data statistics are given in Table S1. Phase determination of the structure amplitudes was achieved by molecular replacement. For the 10-**2Ap**/T·M.TaqI·AETA complex, the structure of the 10-A/T·M.TaqI·AETA complex[13] excluding the target adenine, AETA, and water molecules was used as the initial model. The structure of the 10-**2Ap**/T·M.TaqI·AETA complex with omitted target **2Ap**, partner thymine, AETA, and water molecules then served as the initial model for the 10-**2Ap**/**D**·M.TaqI·AETA complex. Structure refinement was performed with the program CNS[51] and REFMAC5 from the CCP4 suite,[52] and electron density interpretation was carried out using O[53,54] and Coot.[55] Structural figures were generated with DeepView/Swiss-PdbViewer v3.7[56] or VMD 1.8.[57]

Time-Resolved Fluorescence Spectroscopy. Time-resolved fluorescence spectroscopy was performed using the technique of time-

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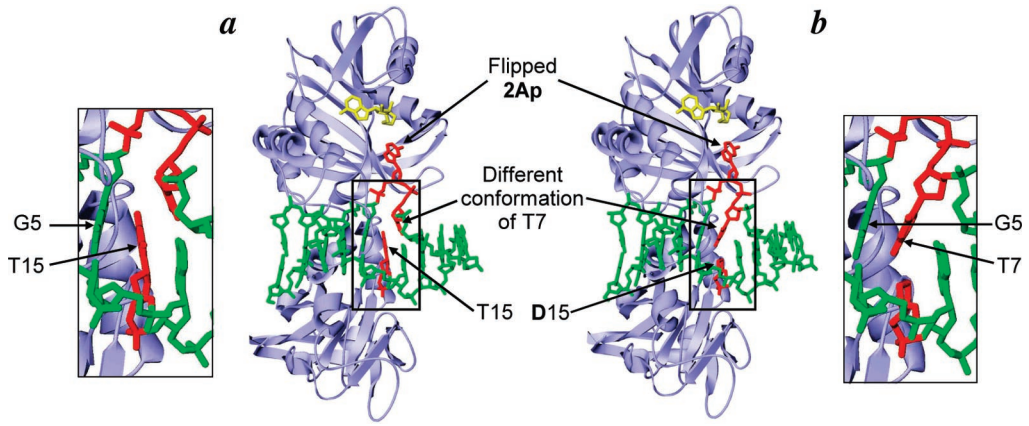

Figure 1. Crystal structures showing **2Ap** in the active site of M.TaqI. (a) Crystal structure (2.4 Å) of the 10-**2Ap**/T·M.TaqI·AETA complex (complex B) with the target base partner thymine (T15) occupying the hole left by the flipped base and π-stacking with G5, the 5′ neighbor of the target base (magnified view in inset on the left). (b) Crystal structure (1.7 Å) of the 10-**2Ap**/**D**·M.TaqI·AETA complex (complex B, **D**15 abasic site analog) with the target base 3′ neighbor thymine (T7) occupying the hole left by the flipped target base and π-stacking with G5 (magnified view in inset on the right). The secondary structural elements of M.TaqI are shown in blue, the cofactor analog AETA in yellow, the DNA in green, and the extra-helical **2Ap** 2′-deoxyribonucleotide, its base partner (T15 or **D**15), and the 3′ neighbor thymine (T7) in red.

correlated single photon counting, using the same general procedure reported previously for the M.HhaI DNA MTase.[48] 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, with 320 nm wavelength. Fluorescence decays were measured in an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics. The instrument response of the system was ~50 ps FWHM. Fluorescence decay curves were analyzed using a standard iterative reconvolution method, assuming a multiexponential decay function. Decays were collected at several emission wavelengths (typically 370, 390, and 410 nm) and analyzed globally using Edinburgh Instruments' FAST software, i.e., they were fitted simultaneously, with lifetimes, τ_i, as common parameters (see Tables S3−S10 in the Supporting Information). The quality of fit was judged on the basis of the reduced chi-square statistic, χ^2, and the randomness of residuals.

Time-Resolved Fluorescence in the Crystalline State. Cocrystals of M.TaqI were too small to allow fluorescence measurements to be made on a single crystal, so fluorescence was collected from many crystals of the same type (see Figure S1 of the Supporting Information). This required the development of a micro handling procedure for separating the small cocrystals from the mother liquor and washing them, as described in the Supporting Information.

Time-Resolved Fluorescence in Solution. Measurements were carried out with duplexes alone (2 μM 14-**2Ap**/T or 14-**2Ap**/**D**), in the presence of M.TaqI (8 μM), and additionally in the presence of AETA (1 mM) in binding buffer. The dissociation constants K_D for the binary complexes 14-**2Ap**/T·M.TaqI and 14-**2Ap**/**D**·M.TaqI are 11 and 0.3 nM, respectively, as determined by competitive steady-state **2Ap** fluorescence titrations.[17] The K_D for the M.TaqI·AETA complex is 0.5 μM and was determined by displacement of the fluorescent cofactor analog 5′-[(3*S*)-3-((*N*-methylanthraniloyl)amino-3-carboxy)-propylthio]-5′-deoxyadenosine from M.TaqI (unpublished result). Thus, the M.TaqI and AETA concentrations used are high enough to ensure quantitative binding of the **2Ap**-labeled duplexes in binary and ternary complexes.

Steady-State Fluorescence Acrylamide Quenching. Titrations were performed by adding increasing amounts of solutions (120 μL) containing **2Ap** 2′-deoxyribonucleotide (50 nM), **2Ap**-labeled duplex (5 μM 14-**2Ap**/T; 300 nM 14-**2Ap**/**D**), or **2Ap**-labeled duplex and M.TaqI (300 nM 14-**2Ap**/T and 1500 nM M.TaqI; 50 nM 10-**2Ap**/**D** and 250 nM M.TaqI) and acrylamide (3.12 M) in binding buffer to solutions (500 μL) of **2Ap** 2′-deoxyribonucleotide (50 nM), **2Ap**-labeled duplex (5 μM 14-**2Ap**/T; 300 nM 14-**2Ap**/**D**), or **2Ap**-labeled duplex and M.TaqI (300 nM 14-**2Ap**/T and 1500 nM M.TaqI; 50 nM 10-**2Ap**/**D** and 250 nM M.TaqI) in binding buffer, respectively. Each data point was background corrected by subtraction of the fluorescence obtained in a parallel titration without the **2Ap** containing compound. Satisfactory fits to the data were obtained by assuming two populations (a and b) of **2Ap** (except for the **2Ap** 2′-deoxyribonucleotide) and using the equation

$$F/F_0 = A_{0a}/(1 + K_a\,[Q]) + A_{0b}/(1 + K_b\,[Q]) \qquad (1)$$

where the fitted parameters $A_{0a,b}$ and $K_{a,b}$ are the fractions of steady-state fluorescence intensities without quencher and the quenching constants, respectively, of the populations a and b. F and F_0 are the fluorescence intensity in the presence and absence of quencher, respectively, and $[Q]$ is the concentration of the quencher acrylamide.

3. Results and Discussion

The cocrystal structures of two **2Ap**-labeled 10 bp DNA duplexes (see Table 1 for sequences) in complex with the DNA adenine-N6 MTase M.TaqI and the nonreactive cofactor analog AETA have been determined. In both duplexes, adenine at the target position of M.TaqI is replaced by **2Ap**. In duplex 10-**2Ap**/T, **2Ap** is paired with thymine, whereas in duplex 10-**2Ap**/**D**, **2Ap** is opposite the abasic site analog 1,2-dideoxy-D-ribose (**D**). The fluorescence decays of both crystalline systems have been measured, together with those of the duplexes and their binary and ternary M.TaqI complexes in solution. Acrylamide steady-state fluorescence quenching has been used to determine the solvent accessibility of **2Ap** in various systems.

3.1. The Environment of 2Ap in the Active Site of M.TaqI.

3.1.1. Crystal Structure of the 10-2Ap/T·M.TaqI·AETA Complex. The complex of 10-**2Ap**/T with M.TaqI and AETA (10-**2Ap**/T·M.TaqI·AETA) shows the same overall structure as the previously determined structure of 10-A/T complexed with M.TaqI and AETA[13] (10-A/T·M.TaqI·AETA). (See Tables S1 and S2 of the Supporting Information for crystallographic details and rms deviations, respectively). Both are indexed in space group $P2_1$ and thus have two symmetry independent, yet similar, complexes in the asymmetric unit (complex A and B).

In 10-**2Ap**/T·M.TaqI·AETA, **2Ap** is flipped into the active site like adenine in 10-A/T·M.TaqI·AETA, and the hole left by the flipped base is occupied by the target base partner

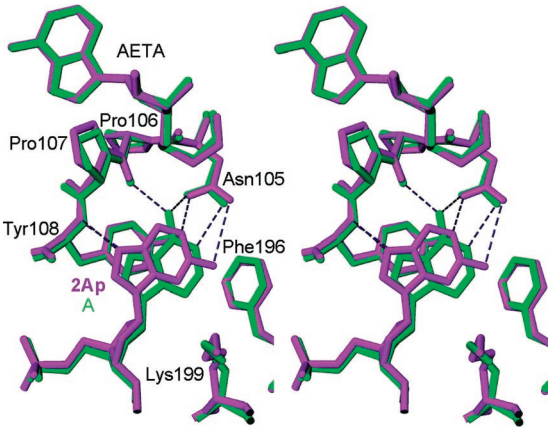

Figure 2. Comparison of the positions of **2Ap** and adenine in the active site of M.TaqI. The stereo representation shows the superposition of **2Ap** in complex A of 10-**2Ap**/T·M.TaqI·AETA (purple) and adenine in complex A of 10-A/T·M.TaqI·AETA (green, PDB ID code 1G38) in the active site of M.TaqI. The hydrogen bonds formed by **2Ap** or adenine are indicated as dotted, black lines. Superposition was performed using all protein backbone atoms resulting in the rms deviations reported in Table S2.

thymine (T15, Figure 1a). As illustrated in Figure 2, **2Ap** is bound in the active site in a very similar fashion to the canonical base adenine. Like adenine, **2Ap** is π-stacked with Tyr108, but due to the different position of the exocyclic amino group, it lacks the face-to-edge aromatic interaction to Phe196 and the hydrogen bond to Pro106/O. A small shift of **2Ap** with respect to adenine brings **2Ap**/N7 closer to Tyr108/N, which probably leads to a stronger hydrogen bond. Asn105 forms different hydrogen bonds to **2Ap** than to adenine. To optimize hydrogen bond distances, the positions of Oδ and Nδ of Asn105 were interchanged with respect to the 10-A/T·M.TaqI·AETA structure[13] and positioned as in the binary complexes of M.TaqI and AdoMet, AdoHcy or Sinefungin.[16] This allows a hydrogen bond to be formed between Nδ of Asn105 and N1 of **2Ap**. Another weak hydrogen bond may be present between N2 of **2Ap** and Oδ of Asn105. Additionally, we interpreted the electron density of the side chain of Lys199 in complex B to represent two different conformations (about 50% each), indicating flexibility of Lys199.

In the previously described ternary DNA·M.HhaI(T250G)·AdoHcy complex containing **2Ap** at the target site,[48] the position of the flipped-out **2Ap** differs from that found for the natural target base cytosine and other bases or analogs at the target position in ternary M.HhaI structures (PDB ID codes 1MHT,[12] 3MHT, 4MHT,[58] 5MHT,[59] 6MHT,[60] 7MHT, 8MHT,[61] 1FJX,[28] 1M0E[62]). Consequently, the local environment reported by the fluorescence response of **2Ap** does not reflect that experienced by the natural base in the M.HhaI active site. In the case of the M.TaqI base-flipped complex, however, the similarity of the positions of **2Ap** and adenine means that the fluorescence decay of **2Ap** will faithfully report the interactions of the natural base within the active site of M.TaqI.

3.1.2. Time-Resolved Fluorescence of 10-2Ap/T·M.TaqI·AETA in the Crystalline State. The 10-**2Ap**/T·M.TaqI·AETA crystals show a multiexponential fluorescence decay, requiring three lifetime components to give a satisfactory fit. This indicates that the ternary complex exists in a number of conformational states in which **2Ap** experiences different interactions and hence different quenching rates. This is similar to the behavior observed previously for DNA-M.HhaI crystalline complexes.[48] The fluorescence lifetimes and their fractional amplitudes are given in Table 2 and plotted in Figure 3a. In a simple interpretation, each lifetime can be considered to represent a distinct conformational state and the fractional amplitude indicates the occupancy of this state. (In fact, each lifetime is likely to represent a distribution of conformations in which **2Ap** experiences similar quenching rates.) It can be seen that although three lifetimes are needed to accurately describe the emitting species, the two shorter 0.93 and 2.3 ns lifetimes (τ_2, τ_3) with fractional amplitudes of 0.46 and 0.51, respectively, account for the vast majority of the population. The minor 8.6 ns lifetime component (τ_4) is due to **2Ap** in an unquenched environment, only 3% of ternary complexes are in this conformation. In the predominant conformations, **2Ap** is substantially quenched by intermolecular interactions. Lifetimes of similar magnitude to these are observed for **2Ap** within the DNA helix, where they are attributed to imperfectly stacked conformations in which quenching of **2Ap** is less efficient than in the highly stacked, major conformation of the duplex that typically has a lifetime of the order of 100 ps or less (*vide infra*).[20,25,47,48] Such "intermediate" lifetimes of $\sim$1$-$3 ns have also been observed with large fractional amplitude on addition of the natural nucleosides to a solution of **2Ap** 2′-deoxyribnuleoside.[45]

The exact nature of the interbase interactions or conformational states responsible for these intermediate lifetimes in DNA or quenched nucleosides remains unclear. However, the interactions responsible for quenching of **2Ap** in the M.TaqI active site are revealed by the crystal structure, which shows that **2Ap** is π-stacked face to face with Tyr108 and is in close proximity to Phe196. As a result of interactions with these aromatic residues (in particular Tyr108), the fluorescence quantum yield for flipped out **2Ap**, relative to free **2Ap** riboside in aqueous solution, is only 0.18. This is significantly different from what was previously observed for the crystalline ternary DNA·M.HhaI(T250G)·AdoHcy complex containing **2Ap** at the target position,[48] where the dominant lifetime is 10.9 ns (fractional amplitude 0.76). In M.HhaI, there are no aromatic residues in proximity to the flipped base and the quantum yield relative to free **2Ap** is 0.9.

The heterogeneity apparent in the fluorescence decay of **2Ap** may be related to the two symmetry independent complexes A and B in the asymmetric unit, which have different positions of Lys199 and slightly different positions of Phe196 in close proximity to **2Ap** (Figure 4a). However, it cannot be ruled out that the lifetime of **2Ap** is affected by conformational heterogeneity that is unresolved in the crystal structure. The long lifetime component has such a small fractional amplitude (0.03) that its corresponding conformations cannot be observed in the crystal structure due to the small contribution to the electron density.

DNA adenine-N6 MTases and DNA cytosine-N4 MTases share the conserved catalytic motif IV (D/N/S)PP(Y/F/W)[63]

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Table 2. Fluorescence Lifetimes (τ_i, $i = 1-4$) and Their Fractional Amplitudes (A_i) for the Crystalline (C) Ternary Complexes (10-**2Ap**/T·M.TaqI·AETA and 10-**2Ap/D**·M.TaqI·AETA), the Free 14-**2Ap**/T and 14-**2Ap/D** Duplexes, Their Binary Complexes with M.TaqI (14-**2Ap**/T·M.TaqI and 14-**2Ap/D**·M.TaqI), and Their Ternary Complexes with M.TaqI and AETA (14-**2Ap**/T·M.TaqI·AETA and 14-**2Ap/D**·M.TaqI·AETA) in Buffered Aqueous Solution (S)[a]

	C	S	τ_1/ ns	τ_2/ ns	τ_3/ ns	τ_4/ ns	A_1	A_2	A_3	A_4 (F_4)	Φ_{rel}[b]
10-**2Ap**/T·M.TaqI·AETA	X		–	0.93	2.3	8.6	–	0.46	0.51	0.03 (0.14)	0.18
14-**2Ap**/T·M.TaqI·AETA		X	–	0.55	1.9	7.3	–	0.54	0.38	0.08 (0.36)	0.15
14-**2Ap**/T·M.TaqI		X	–	0.41	1.9	7.3	–	0.21	0.66	0.13 (0.41)	0.21
14-**2Ap**/T		X	0.03	0.57	2.4	7.8	0.81	0.06	0.09	0.04 (0.53)	<0.055
10-**2Ap/D**·M.TaqI·AETA	X		–	0.92	2.3	7.6	–	0.44	0.48	0.08 (0.29)	0.21
14-**2Ap/D**·M.TaqI·AETA		X	–	0.28	2.8	9.6	–	0.33	0.30	0.37 (0.79)	0.42
14-**2Ap/D**·M.TaqI		X	–	0.23	3.3	9.7	–	0.26	0.18	0.56 (0.89)	0.57
14-**2Ap/D**		X	0.04	0.26	2.5	8.3	0.62	0.18	0.09	0.11 (0.76)	<0.11

[a] Decays collected at several emission wavelengths were analyzed globally to give the reported lifetimes (Tables S3−S10). The fractional amplitudes show little variation with emission wavelength and those for 390 nm (or 380 nm) emission are given. The calculated quantum yield relative to that of free **2Ap** ribonucleoside in water (Φ_{rel}) is also given. For the long lifetime component (τ_4), its fractional contribution to the steady-state fluorescence intensity (F_4) is given, where $F_4 = A_4\,\tau_4/(\Sigma_i\,A_i\,\tau_i)$. [b] For the free duplexes, the Φ_{rel} value quoted is an upper limit as there may be lifetime components shorter than 30 ps present that are beyond the time resolution of our measurements.

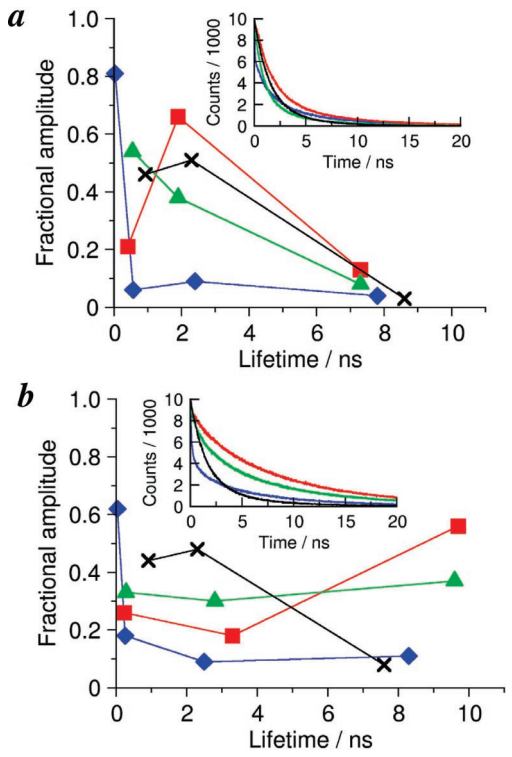

Figure 3. Graphical representation of the response of the **2Ap** fluorescence decay parameters to base flipping. (a) Plots of fractional amplitude (A_i) versus lifetime (τ_i) for the crystalline 10-**2Ap**/T·M.TaqI·AETA complex (black crosses), the ternary 14-**2Ap**/T·M.TaqI·AETA complex in solution (green triangles), the binary 14-**2Ap**/T·M.TaqI complex in solution (red squares), and the free 14-**2Ap**/T duplex in solution (blue diamonds). (Inset) Respective decays following the same color code. (b) Analogous plots for the crystalline 10-**2Ap/D**·M.TaqI·AETA complex, the free 14-**2Ap/D** duplex and its binary and ternary complexes in solution, following the same color code. The connecting lines are for illustrative purposes only.

where the aromatic residue in M.TaqI is Tyr108. Interestingly, these enzymes have overlapping target base specificities.[64,65] The fact that adenine can be methylated by a DNA cytosine-N4 MTase proves that it can be positioned in a catalytically competent conformation within the active site, probably similar to the target adenine in DNA adenine-N6 MTases. Thus, it is likely that **2Ap** takes a similar position within motif IV of DNA cytosine-N4 MTases as in the present 10-**2Ap**/T·M.TaqI·AETA structure. Additionally, RNA adenine-N6 MTases, which can double methylate their target adenine at N6, possess a very similar catalytic motif IV (N/S)(I/L/V)P(Y/F).[66] So it is expected that **2Ap** will be subject to similar quenching interactions in the active sites of other DNA or RNA adenine-N6 MTases as well as DNA cytosine-N4 MTases.

3.1.3. Time-Resolved Fluorescence of 10-2Ap/T and its Binary or Ternary Complexes with M.TaqI in Solution. For the studies in solution, we used a 14 bp DNA duplex exhibiting the same base pairs surrounding **2Ap**-T as in the 10 bp duplex in the crystals: three base pairs at the 5′ side and two base pairs at the 3′ side of **2Ap** are identical (Table 1). Additionally, purines and pyrimidines occur at corresponding positions within the two duplexes. The lifetimes of the systems in solution, which spanned the free DNA 14-**2Ap**/T, the binary 14-**2Ap**/T·M.TaqI complex, and the ternary 14-**2Ap**/T·M.TaqI·AETA complex, are listed in Table 2 and graphically summarized in Figure 3a.

The fluorescence decay for the free 14-**2Ap**/T duplex is multiexponential, requiring four lifetime components to give a satisfactory fit (Table 2 and Figure 3a), indicating the existence of four conformational states, as seen in previous studies of **2Ap**-labeled DNA.[20,47,48] The predominant, very short lifetime (τ_1) of 30 ps with a fractional amplitude of 0.81 is characteristic of **2Ap** in a fully stacked conformation, in which it is subject to efficient charge-transfer quenching from its adjacent guanine stacking partner.[67,68] The intermediate lifetimes (τ_2 and τ_3, imperfectly stacked conformations) constitute 15% of the population, whereas the fraction exhibiting the longest lifetime (τ_4) corresponding to solvent-exposed, extra-helical **2Ap**, as confirmed by acrylamide quenching experiments (*vide infra*), accounts for only 4% of the population.

Binding of M.TaqI to 14-**2Ap**/T results in a profound change in the **2Ap** fluorescence decay, as shown in Table 2 and Figure 3a. The short lifetime (τ_1) attributed to fully stacked **2Ap** in DNA is completely absent in the binary complex. This response to base flipping is much clearer than that recently seen for the T250G mutant of M.HhaI, for which ∼16−19% of a short lifetime (∼150 ps) remained in the binary and ternary complexes in solution.[48]

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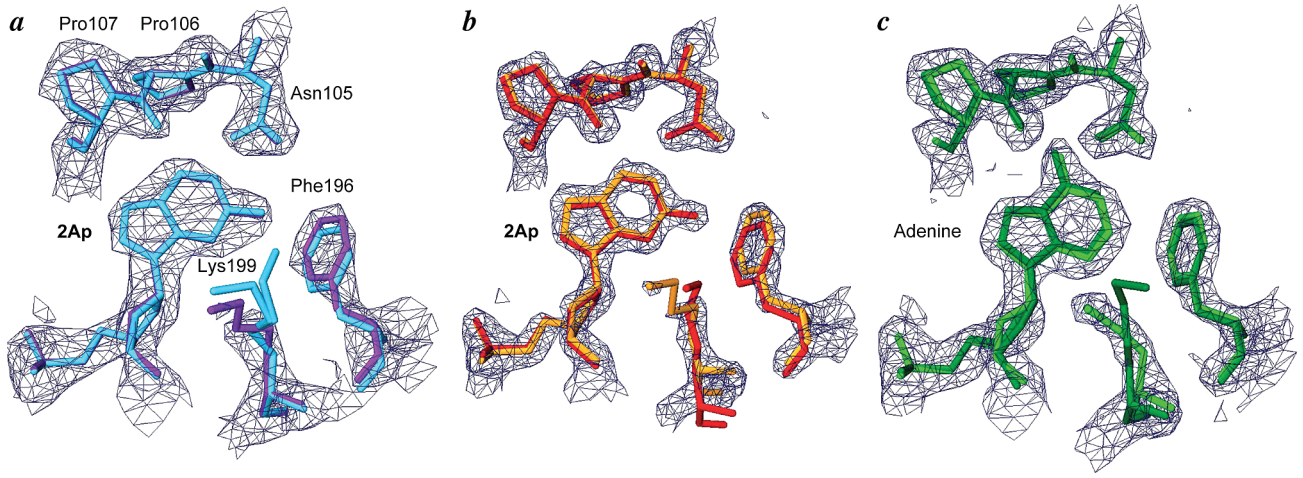

Figure 4. Comparison of the active site structures of complex A and complex B, for 10-**2Ap**/T·M.TaqI·AETA, 10-**2Ap**/**D**·M.TaqI·AETA and 10-A/T·M.TaqI·AETA. (a) **2Ap** and selected active site residues of complex A (purple) are superposed on those of complex B (light blue), from the 2.4 Å crystal structure of the ternary 10-**2Ap**/T·M.TaqI·AETA complex. The electron density (1.5 σ) is shown for complex A, only. (b) The same superposition for complex A (orange) and B (red) of 10-**2Ap**/**D**·M.TaqI·AETA (1.7 Å resolution, electron density at 2.0 σ). (c) The same superposition for complex A (light green) and B (dark green) of 10-A/T·M.TaqI·AETA (2.0 Å resolution, electron density at 1.5 σ, PDB ID code 1G38). Superposition of the complexes A and B, respectively, was performed using all protein backbone atoms resulting in the rms deviations reported in Table S2. **2Ap** and the NPPY motif (of which Tyr108 is omitted for clarity) fit almost perfectly, whereas small differences between complexes A and B can be observed for Phe196 and Lys199.

Comparison of the decay parameters of the binary and ternary complexes in solution shows the effect of binding of the cofactor analog, AETA. Although the lifetimes are almost identical, there is a difference in the fractional amplitudes. This suggests that essentially the same conformations exist in both complexes, but binding of AETA changes the relative population of conformational states: the population of the most quenched conformation (τ_2) increases, whereas the populations of the less quenched conformations (τ_3 and τ_4) decrease.

The similarity between the decay parameters of the ternary 14-**2Ap**/T·M.TaqI·AETA complex in solution and the crystalline 10-**2Ap**/T·M.TaqI·AETA complex, as illustrated graphically in Figure 3a, shows that the fluorescence response of **2Ap** provides a definitive indication of base flipping by M.TaqI in solution. The lifetimes, although clearly indicative of similar environments, are somewhat shorter in solution than in the crystal. This could be due to greater mobility of **2Ap** within the active site and is in line with the observation that the excited-state decay of **2Ap** in duplexes is influenced by base dynamics[67,68,69] but could also indicate that **2Ap** partly occupies imperfectly π-stacked positions within DNA in the M.TaqI complexes in solution. Nevertheless, the active site of the ternary M.TaqI complex in solution necessarily needs to be populated by **2Ap** to crystallize in the observed structure.

3.2. Influence of the Target Base Partner on Base Flipping. The target base partner thymine is believed to have a key role in base flipping by M.TaqI for two reasons, (i) stabilization of the apparent abasic site left by the target base after base flipping[13,17] and (ii) pushing (mediated by domain closure of M.TaqI) the target base out of the DNA.[18] To gain deeper insight into the mechanistic role of the target base partner, we investigated the interaction of M.TaqI with a duplex, 10-**2Ap**/**D** (sequence shown in Table 1), in which **2Ap** is placed at the target position and opposite to the chemically stable abasic site analog 1,2-dideoxy-D-ribose, **D**.

3.2.1. Structure and Time-Resolved Fluorescence of the Crystalline 10-2Ap/D·M.TaqI·AETA Complex. The overall cocrystal structure of the complex of 10-**2Ap**/**D** with M.TaqI and AETA (10-**2Ap**/**D**·M.TaqI·AETA) is again very similar to that of 10-A/T·M.TaqI·AETA, with two symmetry independent complexes (A and B) in the asymmetric unit (see Table S1 for crystallographic details and S2 for rms deviations). In this structure, **2Ap** is also found in the active site and in the same position as in the 10-**2Ap**/T·M.TaqI·AETA structure (Figure 1b and Figure 4b), which indicates that the partner thymine is not obligatory for target base flipping.

In complex A, no major structural differences are observed compared to 10-**2Ap**/T·M.TaqI·AETA and the hole left by the flipped base is simply filled by water molecules. However, a profound structural difference is observed in complex B, where the hole left by the flipped base (that is occupied by the partner thymine, T15, in the 10-A/T·M.TaqI·AETA and 10-**2Ap**/T·M.TaqI·AETA structures) is filled by the 3′ neighbor thymine (T7) of the target base. A similar base shift has been observed in the DNA-protein cocrystal structure of the DNA cytosine-C5 MTase M.HaeIII.[70] As illustrated in Figure 1b (see also Figure S2 of the Supporting Information for details), T7 is π-stacked to the 5′ neighbor guanine (G5) of the extra-helical target base in a similar fashion to the partner thymine (T15) in the 10-A/T·M.TaqI·AETA or 10-**2Ap**/T·M.TaqI·AETA cocrystal structures. The T7 shift could be an alternative way to stabilize the apparent abasic site formed upon target base flipping. Such a T7 shift is also observed in the analogous cocrystal structure with a duplex containing the canonical target adenine opposite to the abasic site analog (10-A/**D**·M.TaqI·AETA, unpublished results).

The decay parameters of the ternary crystalline 10-**2Ap**/**D**·M.TaqI·AETA complex (Table 2 and Figure 3b) are similar to those of the analogous 10-**2Ap**/T·M.TaqI·AETA complex, indicating that **2Ap** experiences similar quenching interactions in the active site. This spectroscopic result mirrors the similar environments of **2Ap** observed in the two crystal structures (Figure 4).

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3.2.2. Time-Resolved Fluorescence of 10-2Ap/D and its Binary and Ternary Complexes with M.TaqI in Solution. The fluorescence decay parameters of the free 14-**2Ap**/**D** duplex resemble those of free 14-**2Ap**/T. The very short lifetime (τ_1) has a lower fractional amplitude in the free 14-**2Ap**/**D** duplex (0.62) than in the free 14-**2Ap**/T duplex (0.81), and the long lifetime (τ_4) has a greater amplitude (0.11 compared with 0.04). Thus, in the absence of the partner thymine, **2Ap** has a greater tendency to exist in an extra-helical state, but the major fraction is still found in a fully π-stacked conformation. A similar trend has been observed before[25] and probably reflects the missing Watson−Crick-like hydrogen bonds and a lack of continuous base stacking in the complementary strand.

Most interestingly, the very short lifetime component is also absent in the 14-**2Ap**/**D**·M.TaqI and 14-**2Ap**/**D**·M.TaqI·AETA complexes in solution (Table 2), demonstrating that **2Ap** has been flipped out of the duplex and confirming that the partner thymine is not essential for base flipping in these complexes. Although it should be thermodynamically easier to flip **2Ap** from the 14-**2Ap**/**D** than from the 14-**2Ap**/T duplex, the time-resolved fluorescence data clearly show that the major fraction of **2Ap** is π-stacked in 14-**2Ap**/**D**, and these π-stacking interactions need to be overcome during the base flipping process. However, in the 14-**2Ap**/**D** duplex, the partner thymine is missing and, thus, it cannot be utilized to actively push out the target base *via* a M.TaqI-mediated DNA compression. In fact, steady-state fluorescence stopped-flow data indicate that M.TaqI-induced **2Ap** flipping from 14-**2Ap**/**D** is more than 2-fold faster than from 14-**2Ap**/T (unpublished results). DNA compression occurs *perpendicular* to the helix axis whereas the 3′ neighbor thymine (T7) shift observed in complex B of the 10-**AP**/**D**·M.TaqI·AETA structure is almost *parallel* to the helix axis. Thus, the 3′ neighbor thymine (T7) is not expected to replicate/rescue a pushing function of the partner thymine. However, the T7 shift could lead to a stabilization of the extra-helical target base by binding into the cavity left in the DNA. So the role of the target base partner seems less likely to be part of an active push mechanism, but rather to stabilize the extra-helical target base conformation by binding to the apparent abasic site after target base flipping.

Apart from the absence of the short lifetime, the decay parameters of the 14-**2Ap**/**D** complexes in solution differ significantly from those of the crystalline 10-**2Ap**/**D**·M.TaqI·AETA complex. In solution, there is a highly populated unquenched conformation (τ_4, fractional amplitude 0.37 in the ternary and 0.56 in the binary complex) with lifetimes of 9.6−9.7 ns, which is in contrast to the crystalline complex in which the unquenched conformation accounts for only 8% of the population. Thus, the crystalline system, in which **2Ap** is located in the M.TaqI active site, appears to represent only a subset of the conformations present in solution; there exists at least another highly populated conformation in solution in which **2Ap** has been flipped out of the duplex (absence of τ_1) but exhibits a long lifetime and, thus, is not located within the quenching environment of the active site of M.TaqI. In this respect, the 14-**2Ap**/**D** complexes in solution also differ from the 14-**2Ap**/**T** complexes in solution; the latter have much lower fractional amplitudes of the long lifetime component and presumably exist predominantly in conformations with **2Ap** located in the active site. Thus, the target base partner thymine appears to be important (albeit not obligatory) for delivering the target base into the active site of M.TaqI and avoiding catalytically incompetent conformations (*vide infra).*

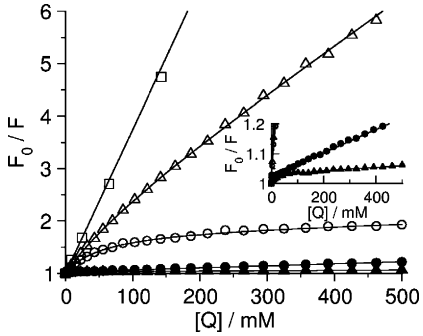

Figure 5. Stern−Volmer plots of fluorescence quenching of **2Ap** by acrylamide (Q). The extent of quenching follows the order: **2Ap** 2′-deoxyribonucleotide (□) ≫ 14-**2Ap**/**D** (△) ≫ 14-**2Ap**/T (○) > 14-**2Ap**/T·M.TaqI (●) ∼ 14-**2Ap**/**D**·M.TaqI (▲). (Inset) Stern-Vollmer plots of 14-**2Ap**/T·M.TaqI and 14-**2Ap**/**D**·M.TaqI magnified. The lines are fits of eq 1 to the data points.

3.3. Evidence for an Alternative Binding Site. We were interested in further characterizing the conformation(s) within the M.TaqI complexes, in which **2Ap** exhibits a long lifetime, approximating that of the free **2Ap** (2′-deoxy)ribonucleoside in solution. It is important to be aware that the steady-state fluorescence intensity is dominated by this long lifetime component; even in the unbound 14-**2Ap**/T duplex, where it constitutes only 4% of the emitting population, it is responsible for 53% of the steady-state fluorescence intensity (Table 2). In the 14-**2Ap**/**D** duplex 76% (fractional amplitude 0.11), in the binary 14-**2Ap**/T·M.TaqI complex 41% (fractional amplitude 0.13), and in the binary 14-**2Ap**/**D**·M.TaqI complex even 89% (fractional amplitude 0.56) of the steady-state fluorescence in solution are caused by the long lifetime component.

To establish the solvent accessibility of **2Ap**, we performed steady-state fluorescence acrylamide quenching experiments with the free duplexes and their respective complexes with M.TaqI and compared the results to free **2Ap** 2′-deoxyribonucleotide. As can be inferred from the Stern−Volmer plots in Figure 5, the extent of quenching of **2Ap** decreases in the order **2Ap** 2′-deoxyribonucleotide ≫ 14-**2Ap**/**D** ≫ 14-**2Ap**/T > 14-**2Ap**/T·M.TaqI ∼ 14-**2Ap**/**D**·M.TaqI. Satisfactory fits to the data were obtained by assuming two populations of **2Ap**, exhibiting different acrylamide quenching constants, in each case except for the **2Ap** 2′-deoxyribonucleotide (single population). The results are listed in Table 3. For the free duplexes, 14-**2Ap**/T and 14-**2Ap**/**D**, the percentage of steady-state fluorescence exhibiting the larger quenching constant (similar to that for the free **2Ap** 2′-deoxyribonucleotide) is in good agreement with the percentage of steady state intensity due to the long lifetime component obtained from time-resolved fluorescence measurements, as shown in Tables 3 and 2: 47% vs 53% for 14-**2Ap**/T and 70% vs 76% for 14-**2Ap**/**D**. In agreement with the results obtained by Stivers[24] and Rachofsky et al.,[25] these results demonstrate that in the long-lifetime conformations of 14-**2Ap**/T and 14-**2Ap**/**D**, **2Ap** is solvent-exposed.

In contrast, the 14-**2Ap**/T·M.TaqI and 14-**2Ap**/**D**·M.TaqI complexes behaved very differently. The percentage of steady-state fluorescence from the populations of **2Ap** with the larger quenching constant is almost negligible (∼3%), although the conformations exhibiting the long lifetime contribute 41% and

Table 3. Fitting Results of the Acrylamide Quenching Titration Curves from Figure 5 Assuming Two Different Populations of **2Ap** (a and b) in All Cases except for Free **2Ap** 2′-Deoxyribonucleotide (One Population)[a]

	K_a/M^{-1}	K_b/M^{-1}	$A_{0a}/\%$	$A_{0b}/\%$
2Ap	26.9 ± 0.9		100.0 ± 1.0	
14-**2Ap**/T	33.2 ± 2.0	0.17 ± 0.04	46.7 ± 0.9	53.3 ± 1.0
14-**2Ap**/**D**	24.8 ± 0.7	3.57 ± 0.20	70.1 ± 1.5	29.9 ± 1.6
14-**2Ap**/T·M.TaqI	132.6 ± 42.3	0.38 ± 0.006	2.7 ± 0.2	97.3 ± 0.2
14-**2Ap**/**D**·M.TaqI	58.7 ± 11.2	0.05 ± 0.005	3.3 ± 0.2	96.7 ± 0.2

[a] $A_{0a,b}$ and $K_{a,b}$ are the percentage contributions to the steady-state fluorescence intensities without quencher and the quenching constants, respectively, of the populations a and b. The larger the quenching constant $K_{a,b}$ the more efficient the quenching and the more solvent accessible the population. (Errors are given as 2σ standard deviation obtained from the fits).

89% to the steady-state fluorescence in 14-**2Ap**/T·M.TaqI and 14-**2Ap**/**D**·M.TaqI, respectively. Therefore, in the long-lifetime conformations of the M.TaqI-bound duplexes, **2Ap** is not solvent exposed but exists in an unquenched, solution-like, polar environment, which is shielded by the enzyme. This leads us to propose an alternative binding site for the flipped base in M.TaqI, that is extra-helical from DNA but is not quenched by Tyr108 within the active site. Such an alternative binding site (exo site) has recently been identified for the base flipping hOGG1 DNA glycosylase with non cognate DNA by X-ray crystallography.[71] A possible functional role of such an exo site in M.TaqI could be to preferentially bind methylated target adenine, providing a check point and avoiding double-methylation of adenine residues. Double-methylation is found for adenine-specific RNA di-MTases, however, in DNA, it would abolish Watson−Crick base pairing and could introduce unwanted mutations.

4. Conclusions

The crystal structures of the ternary M.TaqI complexes with both DNA duplexes, 10-**2Ap**/T and 10-**2Ap**/**D**, show that **2Ap** is flipped into the active site of M.TaqI and occupies a position similar to that found previously for the natural target base, adenine.[13] Thus, **2Ap** can be considered an accurate mimic of the natural target base adenine for M.TaqI and most likely for other DNA and RNA adenine-N6 or DNA cytosine-N4 MTases containing the conserved catalytic motif IV as well. The fluorescence of **2Ap** is strongly quenched in the M.TaqI active site, mainly as a result of π-stacking interaction with the conserved aromatic amino acid side chain of Tyr108. Thus, base flipping is not synonymous with elimination of quenching, as is often assumed. Conformational heterogeneity evident in the fluorescence decay of **2Ap** in the active site can be related to structural heterogeneity in the crystal associated with the two symmetry-independent M.TaqI complexes in the asymmetric unit. The fluorescence decay parameters of the ternary 14-**2Ap**/T complex in solution resemble those of the crystalline complex, indicating that **2Ap** is also flipped into the active site of M.TaqI in solution. Base flipping by M.TaqI is accompanied by the complete loss of the very short lifetime component that is characteristic of fully stacked intra-helical **2Ap** in DNA, which is far clearer than observed previously for M.HhaI.[48] The fluorescence decay of **2Ap** provides a clear signal of base flipping by M.TaqI and will be useful for studying base flipping in other DNA adenine-N6 and DNA cytosine-N4 MTases.

Two main functions in base flipping have been attributed to the target base partner: (i) stabilization of the extra-helical target base conformation by occupying the resulting apparent abasic site in DNA and (ii) pushing the target base out of the DNA helix upon domain closure of M.TaqI. The crystal structure of the 10-**2Ap**/**D**·M.TaqI·AETA complex and time-resolved fluorescence data of binary and ternary 14-**2Ap**/**D** complexes in solution show that base flipping can occur even in the absence of the partner thymine, casting doubt on these functional roles. However, the crystal structure shows that stabilization of the extra-helical target base can be replicated by repositioning of the 3′ neighbor thymine into the apparent abasic site formed after base flipping whereas this 3′ neighbor shift is not expected to exert a push on the target base because it occurs perpendicular to the domain closure of M.TaqI.

The fluorescence decays of the binary and ternary 14-**2Ap**/**D** complexes in solution show that, although **2Ap** is entirely flipped out of the DNA, a substantial fraction of this extra-helical **2Ap** is not located in the quenching environment of the active site of M.TaqI. Acrylamide quenching experiments suggest the existence of an alternative binding site in which **2Ap** is buried within M.TaqI and inaccessible to the solvent. This site, which could be an intermediate in the flipping pathway, is also occupied by **2Ap** in the 14-**2Ap**/T complexes but to a much lesser extent. Thus, although not essential for pushing the target base out of the helix, the target base partner thymine is important in delivering the target base into the active site.

Data Deposition. The coordinates and structure factors for the complexes have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 2IBS and 2IBT).

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Supporting Information Available: General methods, materials, crystallization conditions, micro handling procedure for fluorescence measurement of small cocrystals, Figure S1 of cocrystals used for time-resolved fluorescence measurement, Figure S2 showing the 3′ neighbor thymine shift in more detail, Table S1 of crystallographic data and refinement results, Table S2 comparing rms deviations of M.TaqI backbone atoms in various cocrystal complexes, Tables S3−S10 of detailed fluorescence decay parameters, and two crystallographic information files (CIF) describing the two X-ray cocrystal structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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