DNA–Protein Interactions

Polycyclic Aromatic DNA-Base Surrogates: High-Affinity Binding to an Adenine-Specific Base-Flipping DNA Methyltransferase**

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DNA methylation is an important biological event that serves diverse cellular functions such as protection against endogenous restriction endonucleases, direction of DNA-mismatch repair, as well as regulation of gene expression and DNA replication.[1] DNA methylation is catalyzed by DNA methyltransferases (MTases), which bind to specific DNA sequences and transfer a methyl group from *S*-adenosyl-L-methionine to the exocyclic amino groups of adenine or cytosine or to C5 of cytosine.[2] Interestingly, methylation is commenced by flipping the target nucleotide completely out of the helix.[3] The energy cost for disrupting Watson–Crick hydrogen bonds and base-stacking interactions is mainly compensated by specific binding of the target bases within the active sites of the enzymes and by stabilizing the formed apparent abasic site. Three different mechanisms of abasic-site stabilization have been observed in crystal structures of DNA MTases complexed with DNA. The cytosine-specific DNA MTases M·*Hha*I[4] (Figure 1A) and M·*Hae*III[5] (Figure 1B) either only insert an amino acid side chain into the opened space or insert an amino acid side chain in addition to rearranging the base pairing. This is in contrast to the adenine-specific DNA MTase M·*Taq*I[6] (Figure 1C). In this case, the formed unpaired partner base is inserted into the opened space, resulting in interstrand stacking.

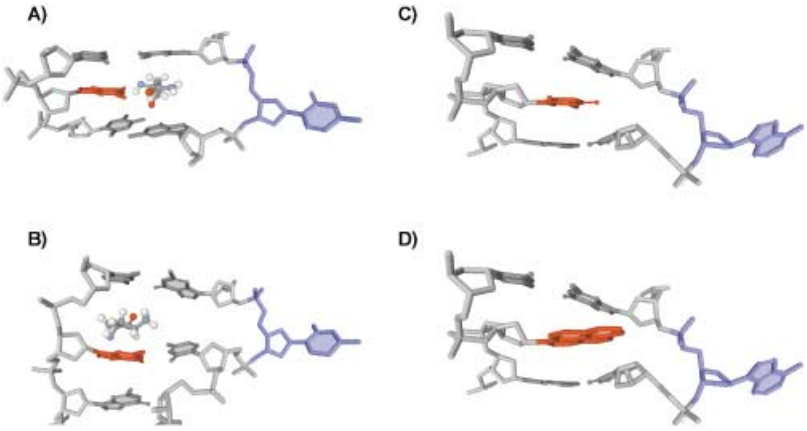

Figure 1. DNA structures found in MTase–DNA complexes with the target extrahelical nucleotide in blue and the orphaned nucleobase in red. A) In the M·*Hha*I structure, Gln237 (represented as a ball-and-stick model) partially occupies the opened space after cytosine flipping and forms hydrogen bonds with the orphaned guanine. B) M·*Hae*III inserts Ile221 (represented as a ball-and-stick model) into the DNA, and the orphaned guanine pairs with the 3′-cytosine that flanks the flipped target cytosine. C) In M·*Taq*I, the orphaned thymine is displaced towards the center of the DNA helix. As a result, interstrand stacking with the 5′ neighboring base of the target adenine occurs. D) A modeled replacement of the orphaned thymine in the M·*Taq*I-DNA structure suggests that aromatic base surrogates such as pyrene (red) fit well into the cavity left by the flipped target adenine and potentially improve interstrand stacking. This filling of the apparent abasic site is likely to contribute to a tightening of the enzyme–DNA complex.

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Inspection of the crystal structure of M·*Taq*I suggested that interstrand stacking could be improved by replacing the partner thymine with larger aromatic DNA-base surrogates. It was expected that more complete filling of the apparent abasic site would lead to tightening of the M·*Taq*I–DNA complex (Figure 1D). In addition, we imagined that a polycyclic base surrogate could destabilize the innerhelical conformation of the opposing target adenine. The DNA MTase could thus bind to double-stranded DNA containing an unstacked target base without having to compensate for the energy required for disrupting Watson–Crick hydrogen bonds and base-stacking interactions. These hypotheses were investigated with DNA substrates that contained pyrenyl, 1-naphthyl, 2-naphthyl, acenaphthyl, and biphenyl residues (**a**–**e**, Scheme 1) opposite to the target position within the recognition sequence of M·*Taq*I.

The synthesis of the required known building blocks **6a–c** and of the new phosphoramidites **6d** and **6e** relies upon the formation of a C-glycosidic bond, which we[7] and others[8] performed by employing the 2-deoxyribose donor **1**[9] and cadmium–organic reagents such as **3a–e** as glycosyl acceptors (Scheme 1).[10] We sought an alternative C-glycosylation

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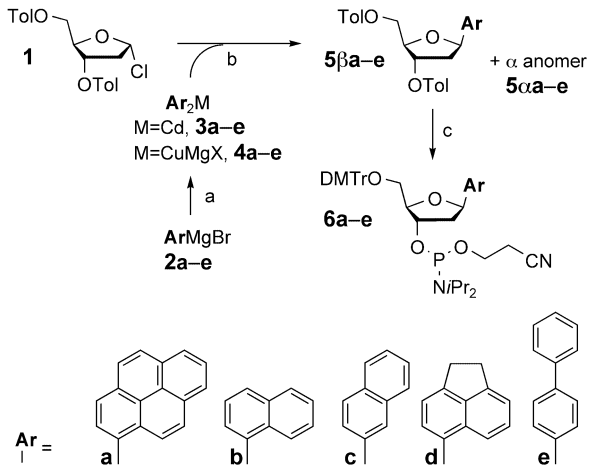

Scheme 1. a) M = Cd: $CdCl_2$, THF, 40–50 °C, 2–3 h; M = CuMgX: CuI, THF, 0 °C, 5 min; b) M = Cd: 0 °C–reflux, THF, 1–2 h; M = CuMgX: THF, 25 °C, 30 min; c) 1) NaOMe, MeOH, 4 h; 2) DMTr-Cl, DMAP, EtN*i*Pr$_2$, pyridine, CH_2Cl_2, 8 h; 3) (*i*Pr$_2$N)$_2$POCH$_2$CH$_2$CN, tetrazole, CH_2Cl_2, MeCN, 1–2 h, 59% (**6a**), 67% (**6b**), 40% (**6c**), 80% (**6d**), 51% (**6e**). DMTr = dimethoxytrityl, DMAP = dimethylaminopyridine.

method with the aims of replacing the toxic cadmium and improving the sometimes low glycosylation yields. Organocuprates are mild and versatile C-nucleophiles in the coupling with reactive alkyl chlorides.[11] Indeed, the C-glycosylation of the Normant reagents **4a–e** proved superior to the reaction with the organocadmium reagents **3a–e** in terms of both synthetic practicability and yields (Table 1). The synthesis of the building blocks **6a–e** was completed after removal of the tolyl protecting groups from **5a–e**, regioselective introduction of the trityl protecting group, and final phosphitylation.

Table 1: Yields of the C-glycosylation reactions.[a]

Organometallic reagent	5a	5b	5c	5d	5e
Ar$_2$Cd, **3a–e**	72% (4:1)	67% (4:1)	47% (2:1)	47% (2:1)	41% (2:1)
Ar$_2$CuMgX, **4a–e**	74% (3:1)	81% (4:1)	74% (3:1)		75% (2:1)

[a] The α/β ratio is given in parentheses.

The phosphoramidites **6a–e** were used in the automated solid-phase synthesis of oligonucleotides **7a–e** (Table 2).[7] First, we investigated whether the presence of the base surrogates could be tolerated in duplex DNA. The modified oligonucleotides **7a–e** were hybridized with the complementary strand **8A**, which contains a target adenine opposite to the base surrogates within the 5′-TCGA-3′ recognition sequence of M·*Taq*I (Table 2). In all cases the melting curves exhibited a sigmoid behavior. Substitution of thymine in **7T** by the aromatic base surrogates in **7a–e** led to a decrease in the melting temperature by 4.9–8.7 K.

After having secured the integrity of the duplexes **7a–e·8A**, their binding affinities to the M·*Taq*I DNA MTase were investigated in solution and compared with the binding

Table 2: Thermal stability of duplexes and binding affinities to the adenine-specific DNA MTase M·*Taq*I.[a]

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5'-GCCGC T C G dRibX TGCCG-3'   8A, 8b
3'-CGGCG A G C dRibY ACGGC-5'   7T, 7C, 7G, 7A, 7a-e
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Y	X = adenine, **8A**		X = H, **8b**	
	T_M [°C][b]	K_A [10^9 M^{-1}][c]	T_M [°C][b]	ΔT_M [K][d]
thymine, **7T**	65.9 ± 0.2	0.05 ± 0.02	49.8 ± 0.1	0.0
cytosine, **7C**	55.7 ± 0.1	0.03 ± 0.02	50.6 ± 0.1	+0.8
guanine, **7G**	61.0 ± 0.2	0.03 ± 0.02	48.7 ± 0.2	−1.1
adenine, **7A**	56.1 ± 0.2	0.04 ± 0.02	52.5 ± 0.2	+2.7
1-pyrenyl, **7a**	61.0 ± 0.3	20 ± 10	63.6 ± 0.4	+13.8
4-biphenyl, **7e**	57.5 ± 0.3	3.0 ± 1.0	59.0 ± 0.3	+9.2
acenaphthyl, **7d**	59.0 ± 0.4	2.0 ± 1.0	57.9 ± 0.3	+8.1
1-naphthyl, **7b**	57.2 ± 0.1	2.0 ± 1.0	56.0 ± 0.2	+6.2
2-naphthyl, **7c**	58.0 ± 0.1	1.0 ± 0.5	57.8 ± 0.2	+8.0

[a] The double-stranded recognition sequence of M·*Taq*I is boxed. [b] Measured as denaturation curves at a DNA concentration of 1 μM in a buffered solution (100 mM NaCl, 10 mM NaH_2PO_4, pH 7.0). [c] For experimental details, see Supporting Information. [d] ΔT_M values are based on the T_M value of **8b·7T**.

affinities of the matched duplex **7T·8A** and the duplexes **7C·8A**, **7G·8A**, and **7A·8A**, which contain a mismatched target adenine. The affinities were determined in a new competitive fluorescence binding assay by employing a competitor duplex that contained the fluorescent base analogue 2-aminopurine at the target position.[12,13] Owing to a partial overlap of the spectra of the 2-aminopurine and pyrene fluorophores, this binding assay was not suitable for the pyrene-containing duplex **7a·8A**. Therefore, a pyrene fluorescence binding assay was performed which employed a nonfluorescent duplex oligonucleotide as competitor.[13]

M·*Taq*I binds its unmodified substrate **7T·8A** with an affinity constant of $K_A = 5 \times 10^7$ M^{-1} and the duplexes **7C·8A**, **7G·8A**, and **7A·8A** containing a mismatched target adenine with almost the same affinity (Table 2). This similarity suggests that removal of the Watson–Crick hydrogen bonds within the target base pair has little effect. However, this finding differs from results with the cytosine-specific DNA MTase M·*Hha*I, in which an up to 20-fold enhanced binding affinity was observed with duplexes that contained a mismatched target cytosine.[14]

In contrast to the duplexes with natural nucleobases, duplexes with the naphthyl, acenaphthyl, and biphenyl base surrogates enhanced the binding affinity by a factor of 20–60. The ≈400-fold enhancement of DNA MTase binding affinity determined for the duplex **7a·8A** containing the pyrenyl residue was most remarkable. The observed affinity corresponds to a dissociation constant $K_D = 50$ pM.

In principle, the enhancement of binding affinity to M·*Taq*I could originate from unstacking the target adenine in the duplexes **7a–e·8A**. This possibility was explored in previous investigations.[7] Fluorescence studies suggested that the pyrenyl residue was rather ineffective in disrupting target base stacking. On the contrary, the biphenyl residue, which led to a sevenfold lower affinity to M·*Taq*I, was the most potent base surrogate in unstacking its partner base. These results suggest that although binding of DNA to M·*Taq*I might profit from an unstacked target base, it is not the

decisive determinant for the observed order of binding affinities.

Binding of M·*Taq*I to its target DNA leads to the formation of an apparent abasic site, which is only partially filled by the interstrand stacked partner thymine (Figure 1C). Abasic sites, however, dramatically decrease duplex stability, as exemplified by the substantial decrease in the melting temperature that was observed when the target 2′-deoxyadenosine in **7T·8A** was replaced by the abasic-site analogue 1,2-dideoxy-D-ribose in **7T·8b** ($\Delta T_M = -16.1$ K). This destabilization also occurred with the other natural nucleobases, as evidenced by similar T_M values of duplexes **7C·8b**, **7G·8b**, and **7A·8b**. We explored whether the base surrogates opposite to an abasic site are capable of increasing duplex stability. Indeed, each of the duplexes **7a–e·8b**, which contain an abasic site opposite to any of the five base surrogates, displayed an enhanced thermal stability relative to the duplex **7T·8b** (Table 2). Most importantly, the thermal stabilities of the abasic site duplexes **7a–e·8b** correlate well with the binding affinities of duplexes **7a–e·8A** to M·*Taq*I (Table 2). The pyrene residue conferred the highest abasic-site stabilization and led to the highest binding affinity to M·*Taq*I. Thus, we propose that the pyrenyl residue enhances the M·*Taq*I-binding affinity mainly by an increased compensation of the energy penalty that arises from the enzyme-induced abasic-site formation.

Recently, studies of uracil DNA glycosylase (UDG) and variants with a duplex that contained the pyrenyl residue opposite to an unreactive uracil analogue were reported.[15,16] Similar to the cytosine-specific DNA MTase M·*Hha*I, UDG flips its target base out of the DNA helix and stabilizes the apparent abasic site by insertion of an amino acid side chain. In contrast to our binding studies with M·*Taq*I, only a minor change in the binding affinity to UDG was conferred by the pyrenyl residue, which is in agreement with the different mechanisms of stabilizing the apparent abasic sites. In M·*Taq*I, the pyrenyl residue fills the space formed by target base flipping better than the natural partner thymine, whereas in UDG a steric interference with the inserting amino acid side chain is expected. In line with this interpretation, the weak binding affinity of a UDG variant in which the critical amino acid side chain is deleted could be restored by incorporation of a pyrene nucleotide.[15,16] This comparison between M·*Taq*I and UDG illustrates that duplexes with extended aromatic base surrogates opposite to the target base could be very useful probes to analyze the mechanism of DNA base-flipping enzymes. In addition, the observed high-affinity binding the of duplex with a pyrenyl residue is of interest for the inhibition of adenine-specific DNA MTases from pathogenic bacteria which are essential for bacterial virulence.[17]

In conclusion, we have presented an improved and less toxic C-glycosylation that is expected to provide a reliable access to a variety of nonpolar nucleoside analogues. Our results point out that improved abasic-site stabilization is an important criterion for high-affinity binding to M·*Taq*I. Future work will concern the implementation of this design approach for the development of high-affinity binders to other adenine-specific DNA MTases, an endeavor ultimately aimed at the construction of inhibitors of these interesting enzymes.

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