Functional Roles of the Conserved Aromatic Amino Acid Residues at Position 108 (Motif IV) and Position 196 (Motif VIII) in Base Flipping and Catalysis by the N6-Adenine DNA Methyltransferase from *Thermus aquaticus*†

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ABSTRACT: The DNA methyltransferase (Mtase) from *Thermus aquaticus* (M·*Taq*I) catalyzes the transfer of the activated methyl group of *S*-adenosyl-L-methionine to the N6 position of adenine within the double-stranded DNA sequence 5′-TCGA-3′. To achieve catalysis M·*Taq*I flips the target adenine out of the DNA helix. On the basis of the three-dimensional structure of M·*Taq*I in complex with the cofactor and its structural homology to the C5-cytosine DNA Mtase from *Haemophilus haemolyticus*, Tyr 108 and Phe 196 were suggested to interact with the extrahelical adenine. The functional roles of these two aromatic amino acid residues in M·*Taq*I were investigated by mutational analysis. The obtained mutant Mtases were analyzed in an improved kinetic assay, and their ability to flip the target base was studied in a fluorescence-based assay using a duplex oligodeoxynucleotide containing the fluorescent base analogue 2-aminopurine at the target position. While the mutant Mtases containing the aromatic amino acid Trp at position 108 or 196 (Y108W and F196W) showed almost wild-type catalytic activity, the mutant Mtases with the nonaromatic amino acid Ala (Y108A and F196A) had a strongly reduced catalytic constant. Y108A was still able to flip the target base, whereas F196A was strongly impaired in base flipping. These results indicate that Phe 196 is important for stabilizing the extrahelical target adenine and suggest that Tyr 108 is involved in placing the extrahelical target base in an optimal position for methyl group transfer. Since both aromatic amino acids belong to the conserved motifs IV and XIII found in N6-adenine and N4-cytosine DNA Mtases as well as in N6-adenine RNA Mtases, a similar function of aromatic amino acid residues within these motifs is expected for the different Mtases.

In addition to the normal nucleobases adenine, thymine, guanine, and cytosine, the DNA of most organisms contains the methylated bases C5-methylcytosine (*1, 2*), N6-methyladenine (*3*), or N4-methylcytosine (*4*). These methylated bases are formed by DNA methyltransferases (Mtases)[1] which catalyze the transfer of the activated methyl group from the cofactor *S*-adenosyl-L-methionine (AdoMet) to the C5 carbon of cytosine, the N6 nitrogen of adenine, or the N4 nitrogen of cytosine within their DNA recognition sequences (*5*). Since a particular nucleotide sequence may exist in its methylated or unmethylated form, DNA methylation can be regarded as an increase of the information content of DNA (*6*), which serves a wide variety of biological functions. In prokaryotes DNA methylation is involved in protection of the host genome against endogenous restriction endonucleases, DNA mismatch repair, regulation of gene expression, and DNA replication (*7, 8*). In eukaryotes DNA methylation plays a role in important regulatory processes, such as regulation of gene expression, embryonic development, genomic imprinting, X-chromosome inactivation, and carcinogenesis (*9*).

Apart from these intriguing biological functions of DNA methylation, the DNA Mtases themselves are fascinating enzymes for structure−activity studies. Since the bases are hidden in the interior of the DNA helix, the question arises about how DNA Mtases reach their target bases. An answer

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; DTT, dithiothreitol; *Eco*Dam, N6-adenine DNA methyltransferase Dam from *Escherichia coli*; M·*Eco*RI, M·*Eco*KI and M·*Eco*P15I, N6-adenine DNA methyltransferases from *Escherichia coli* strains RY13, K12, and 15T$^-$, respectively; M·*Eco*P1I, N6-adenine DNA methyltransferases from *Escherichia coli* prophage P1; M·*Hae*III, C5-cytosine DNA methyltransferase from *Haemophilus aegypticus*; M·*Hha*I, C5-cytosine DNA methyltransferase from *Haemophilus haemolyticus*; M·*Mva*I, N4-cytosine DNA methyltransferase from *Micrococcus varians*; M·*Pvu*II, N4-cytosine DNA methyltransferase from *Proteus vulgaris*; M·*Taq*I, N6-adenine DNA methyltransferase from *Thermus aquaticus*; Mtase, methyltransferase; N-DNA Mtases, N6-adenine and N4-cytosine DNA methyltransferases; N-DNA/RNA Mtases, N6-adenine and N4-cytosine DNA as well as N6-adenine RNA methyltransferases; ODN, oligodeoxynucleotide; R·*Taq*I, restriction endonuclease from *Thermus aquaticus*; T4 Dam, N6-adenine DNA methyltransferase Dam from phage T4; Tris, 2-amino-2-hydroxymethyl-1,3-propandiol.

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to this question was recently provided by the three-dimensional structures of the C5-cytosine DNA Mtase M·*Hha*I and M·*Hae*III in complex with DNA (*10, 11*). In these protein−DNA complexes the target cytosines are flipped out of the DNA helix and placed in a cleft within each enzyme, where catalysis takes place. Here an active site cysteine residue, which is conserved among all C5-cytosine DNA Mtases, attacks the double bond at the C6 position of the cytosine ring and activates the C5-carbon for nucleophilic attack of the methyl group from AdoMet. After methyl group transfer the methylated base is liberated by β-elimination of the proton at C5 and the active site cysteine residue (*12, 13*).

In contrast to the well-understood catalytic mechanism of C5-cytosine DNA Mtases, our knowledge about the methylation mechanism of N6-adenine and N4-cytosine DNA Mtases is still very limited. For the N6-adenine DNA Mtase M·*Eco*RI it was demonstrated that the methyl group is transferred directly to N6 and that the reaction proceeds with inversion of configuration of the methyl group (*14, 15*). In addition, a base flipping mechanism was demonstrated for the two adenine-specific DNA Mtases M·*Eco*RI and M·*Taq*I using duplex oligodeoxynucleotides (ODNs) containing the fluorescent base analogue 2-aminopurine at the target positions (*16, 17*). A base flipping mechanism of N6-adenine as well as N4-cytosine DNA Mtases was also suggested on the basis of the crystal structures of M·*Taq*I (Figure 1) and M·*Pvu*II (an N4-cytosine DNA Mtase) in complex with the cofactor (*18, 19*). Both enzymes consist of two domains, which form a positively charged cleft wide enough to accommodate double-stranded DNA. However, modeling B-DNA into these clefts showed that the distance between the target bases and the activated methyl group of the cofactor is too large for a direct methyl group transfer. By rotating the target bases out of the DNA helix toward the cofactor, the distance between the methyl group acceptors and donor can be significantly reduced; hence a base flipping mechanism was proposed for M·*Taq*I and M·*Pvu*II (*19, 20*).

A structural comparison of the N6-adenine DNA Mtase M·*Taq*I with the C5-cytosine DNA Mtase M·*Hha*I showed that the catalytic domains of both enzymes have a very similar fold (*21*). On the basis of this finding, Malone et al. (*22*) performed a structure-guided sequence comparison of N6-adenine and N4-cytosine DNA Mtases, identifying nine conserved sequence motifs, which correspond to the motifs I to VIII and X previously found in C5-cytosine DNA Mtases (*23*). These conserved sequence motifs are also found in N6-adenine RNA Mtases (*22, 24, 25*). In a structural superimposition of the catalytic domains of M·*Taq*I and M·*Hha*I the cofactors and the motifs IV (N/D/S P/I P Y/F/W-motif of N-DNA/RNA Mtases and PCQ-motif of C5-cytosine DNA Mtases containing the catalytic cysteine residue) as well as the motifs VIII (F/Y/W-motif in N-DNA/RNA Mtases and QxRxR-motif in C5-cytosine DNA Mtases) overlay very well, and it was suggested that the extrahelical adenine in M·*Taq*I is located in a position similar to that of the extrahelical cytosine in M·*Hha*I (*21*). Since adenine and cytosine are structurally and chemically very different, amino acid residues with different side chains are expected to interact with the extrahelical target bases in N6-adenine and C5-cytosine DNA Mtases. However, spatially corresponding amino acid residues could have similar functions, and it was

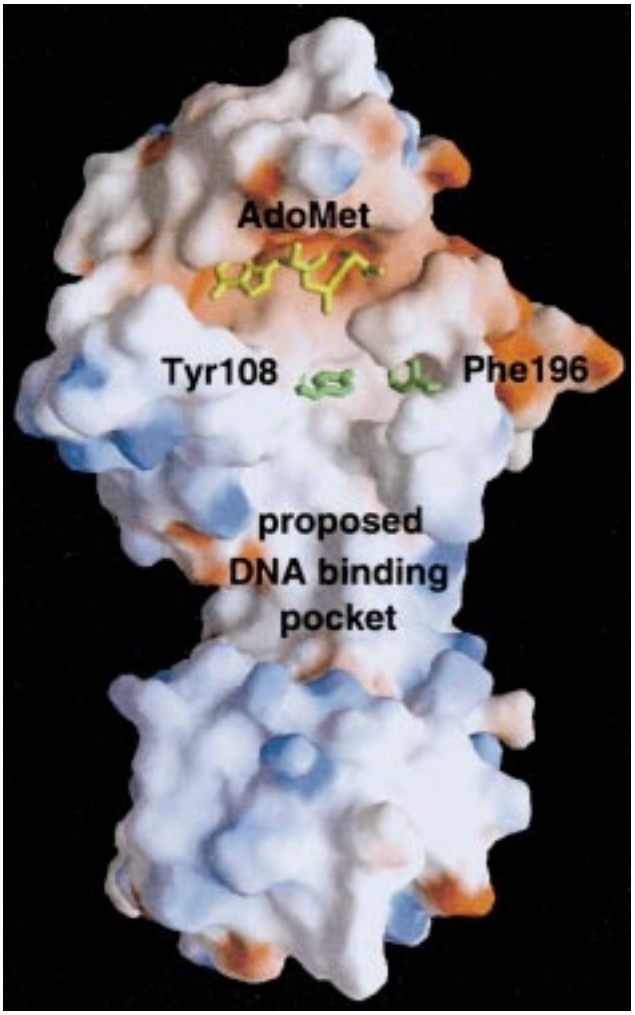


FIGURE 1: Structure of M·*Taq*I in complex with AdoMet (*30*) showing the spatial relationships between Tyr 108 (green), Phe 196 (green), the cofactor (yellow), and the proposed DNA binding pocket. The electrostatic potential surface (positive charge blue, negative charge red, neutral white) was calculated in the absence of Tyr 108, Phe 196, and AdoMet using the computer program GRASP (*54*), and the missing amino acid residues and the cofactor were than added to the structure. It was proposed that DNA binding occurs in the highly positively charged cleft between the two domains (*18*) and that the target adenine interacts with Tyr108 and Phe196 after base flipping (*21, 22*).

postulated that Tyr 108 (motif IV) and Phe 196 (motif VIII) in M·*Taq*I (Figure 1) could be responsible for proper orientation of the extrahelical adenine (*21, 22*). In addition, it was speculated that these two aromatic amino acid residues could be directly involved in the catalytic process. Formally, M·*Taq*I catalyzes the exchange of a proton at the N6-position of adenine within the double-stranded DNA sequence 5′-TCGA-3′ for a methyl group from AdoMet (Scheme 1, ref *26*). If the methyl group would be transferred from AdoMet to N6 of adenine prior to proton release, a positively charged intermediate (transition state) would be formed, which could be stabilized by cation-π interactions with the aromatic side chains of Tyr 108 and Phe 196 (*27*).

To test whether the extrahelical target adenine in M·*Taq*I indeed interacts with Tyr 108 and Phe 196, we undertook a systematic mutational analysis. Tyr 108 was replaced by the aromatic amino acids Phe and Trp as well as by the nonaromatic amino acids Gly and Ala. In addition, Phe 196 was substituted by the aromatic amino acid Trp and the

nonaromatic amino acid Ala. All mutant Mtases were purified to near homogeneity and tested for their catalytic competence in an in vitro protection assay. The most interesting mutant Mtases were further characterized kinetically in an improved steady-state assay, and their ability to bind DNA and to flip the target base was analyzed in a fluorescence assay using a duplex ODN containing the fluorescent base analogue 2-aminopurine at the target position.

MATERIALS AND METHODS

DNA Synthesis and Purification. ODNs were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer, using standard β-cyanoethyl phosphoramidite chemistry, or were purchased from MWG Biotech. 2-Aminopurine and N6-methyladenine phosphoramidites were purchased from Glen Research. For the mutagenesis experiments the following ODNs were synthesized "trityl off" and used without purification (base changes are printed in bold):

5′-AATCCGCCTT**TT**GGCATCGTAG-3′
(**1**, plus strand primer Y108F)

5′-AATCCGCCTT**GG**GGCATCGTAG-3′
(**2**, plus strand primer Y108W)

5′-ATCCGCCT**NNN**GGCATCGTAG-3′
(**3**, degenerate primer Y108)

5′-TGCCCAGGATCAGGTCAAAGG-3′
(**4**, minus strand primer Y108)

5′-CGAGGTT**GC**CCCGCAAAAAAAGGTTAGCGC-3′
(**5**, plus strand primer F196A)

5′-CGAGGTTT**GG**CCGCAAAAAAAGGTTAGCGC-3′
(**6**, plus strand primer F196W)

5′-CCAAGGTAGTATACAGATGTTTTCCC-3′
(**7**, minus strand primer F196)

The following ODNs were synthesized for steady-state kinetic measurements and DNA binding studies (the target sequence is printed in bold, A^{Me} = 6-methyl-2′-deoxyadenosine, 2 = 2-aminopurine-1′-β-D-2′-deoxynucleoside):

5′-GCTGTTGAGATCCAGT**TCGA**
TGTAACCCACTCGTGC-3′ (**8**)

5′-GCACGAGTGGGTTACA**TCGA^{Me}**
ACTGGATCTCAACAGC-3′ (**9**)

5′-GCTGTTGAGATCCAGT**TCG2**
AGTAACCCACTCGTGC-3′ (**10**)

5′-GCTGTTGAGATCCAGT**TCGA**
AGTAACCCACTCGTGC-3′ (**11**)

5′-GCACGAGTGGGTTACT**TCGA^{Me}**
ACTGGATCTCAACAGC-3′ (**12**)

Synthesis and purification of these ODNs as well as formation of duplex ODNs (**8·9**, **10·12**, and **11·12**) were performed as described before (*17*).

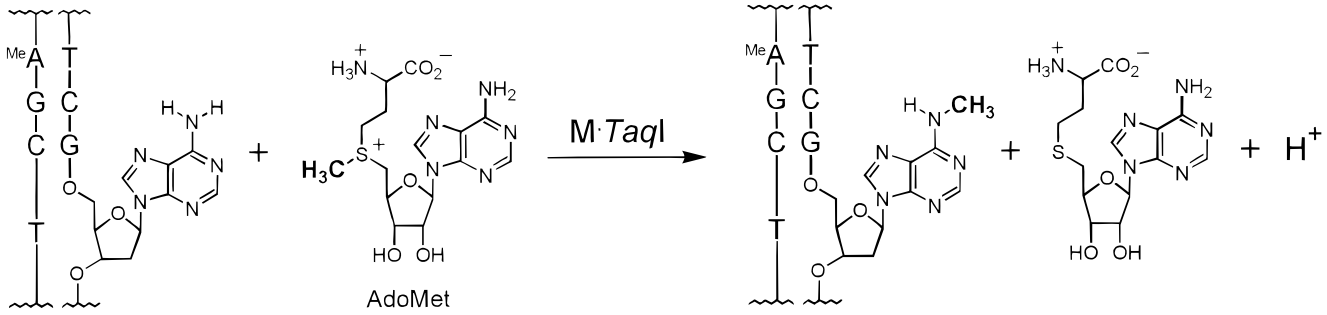
Kunkel Mutagenesis. An expression vector for M·*Taq*I (pAGL15-M13) was kindly provided by Dr. Jack Benner and Dr. Cheryl Baxa, New England Biolabs. The *taqIM* gene from pAGL15-M13 was isolated as 1280 bp *Hind*III/*Nco*I fragment and subcloned by standard procedures (*28*) into a derivative of M13mp19 containing a *Nco*I and *Hind*III restriction site in the polylinker sequence. ODNs **1** and **2** were 5′-phosphorylated, and the Kunkel mutagenesis was performed using the Muta-Gene M13 in vitro mutagenesis kit from Biorad. Plasmids of individual clones were sequenced in the mutagenised region using the Terminator Kit (Perkin-Elmer) and automated DNA sequencing (373 DNA Sequencer, Applied Biosystems). All analyzed clones (10 for the Phe and four for the Trp replacement at postion 108) carried the desired mutations. The mutant genes for Y108F and Y108W were excised with *Hind*III and *Nco*I and subcloned back into the expression vector. Both mutant genes were fully sequenced and contained only the desired base substitutions.

Inverse PCR Mutagenesis. ODNs **3**, **4**, **5**, **6**, and **7** were 5′-phosphorylated, and mutagenesis was accomplished directly in pAGL15-M13. The inverse PCR and subsequent reactions were performed as described before (*29*). Sixty-two individual clones of the base substitution library obtained with the ODN pair **3**/**4** were analyzed in the mutagenised region by DNA sequencing. Eighteen different of the 64 possible codons were found: GGG (15), GGT (6), GGA (5), and GGC (4) for Gly; CGC (5), CGG (3), AGG (3), and CGA (2) for Arg; GAG (7) and GAA (1) for Glu; GCC (2), GCG (1), and GCT (1) for Ala; AAG (3) for Lys; GTG (1) and GTC (1) for Val; TCG (1) for Ser; and CAA (1) for Gln. Thirty-two of the sequenced clones contained only mutations in the targeted codon 108, while the other clones carried additional point mutations, insertions, or deletions within the sequenced fragment of approximately 400 bases. Eight clones of each mutagenesis reaction with the ODN pair **5**/**7** or **6**/**7** were sequenced in the mutagenized region, and all clones contained the desired mutation. However, many of the examined clones carried additional base substitutions and deletions as observed in the base substitution library. Plasmids with the genes for Y108A, Y108G, F196A, and F196W containing only the desired mutation within the initially analyzed region were fully sequenced in the Mtase gene and revealed no further mutations.

Expression and Purification of Wild-Type and Mutant Proteins. The proteins were expressed in *Escherichia coli* cells (ER 2267, New England Biolabs) harboring pAGL15-M13 or the corresponding expression plasmids for the mutant proteins. Protein purification was performed as described before (*17*). The proteins were at least 95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and staining with Coomassie Blue. However, it was found that a fraction of most proteins still contained the cofactor AdoMet, and AdoMet bound to the Mtases was removed as previously described (*30*). Protein concentrations were estimated by the method of Bradford (*31*) (Coomassie Protein Assay Reagent, Pierce) using bovine serum albumin as standard. Concentrations were calculated using a molecular weight of 47 900 g/mol.

DNA Protection Assay. Ten reaction solutions (20 μL) containing a 2-fold serial dilution of Mtase (wild type, Y108F, Y108W, and F196W, 10−0.02 ng; Y108A, Y108G, and F196A, 1000−2 ng), λ-DNA (1 μg, Fermentas), AdoMet (80 μM), bovine serum albumin (100 μg/mL) and reaction buffer (20 mM Tris acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, and 0.01% Triton X-100, pH 7.9, at 25 °C) were incubated at 65 °C for 60

Scheme 1: Reaction Catalyzed by M·*Taq*I



min. Afterward a solution (30 μL) containing R·*Taq*I restriction endonuclease (40 units, Fermentas), bovine serum albumin (100 μg/mL), and buffer (10 mM Tris hydrochloride, 100 mM sodium chloride, 5 mM magnesium chloride, pH 8.0) was added to each reaction, and the incubation at 65 °C was continued for 15 min. Loading buffer (10 μL, 0.25% bromphenol blue, 30% glycerol) was added to each reaction, and aliquots (10 μL) were analyzed for the degree of protection by agarose gel (1%) electrophoresis.

Improved Radioactive Kinetic Assay. [Methyl-^{3}H]-AdoMet (15 Ci/mmol, 550 μCi/mL, 15.2 cpm/fmol) was purchased from Dupont NEN and mixed with nonradioactive AdoMet (Sigma) to reduce the specific radioactivity. Linearity of the assay and thermostability of wild-type and mutant Mtases were tested by incubation of a solution (60 μL) containing Mtase, duplex ODN **8·9** (1.0 μM), [methyl-^{3}H]-AdoMet (20 μM, mixed with a 9-fold excess of nonradioactive AdoMet, 1.5 Ci/mmol), and reaction buffer at 60 °C. Before assaying, the Mtase and DNA were thermally equilibrated in 1.1-fold concentrated reaction buffer (54 μL) at 60 °C for 15 min, and the reaction was started by adding [methyl-^{3}H]-AdoMet (6 μL). Aliquots (10 μL) were removed after different incubation times and transferred into the wells of a microtiter plate filter unit (Event Detect Test Plate, positively charged membrane, Eppendorf) which were filled with a silica DEAE-anion-exchange resin (100 μL, equilibrated with reaction buffer without Triton X-100, Nucleobond AX, Macherey-Nagel) and nonradioactive AdoMet (100 μL, 1 mM). The filter unit was placed in ice to ensure fast quenching of the reaction. After addition of all aliquots the filter unit was placed onto a vacuum unit (Event 4160, Eppendorf), and unreacted [methyl-^{3}H]-AdoMet was washed away with ammoniumhydrogen carbonate buffer (10 times 200 μL, 200 mM). The DNA was eluted with a solution (five times 100 μL) containing sodium chloride (1 M) and potassium hydroxide (50 mM). Scintillation cocktail (5 mL, Ready Gel, Beckmann) was added to the filtrate, and the amount of tritium transferred to the DNA was quantified by scintillation counting (LS6500, Beckman) for 10 min, resulting in a counting error below 2%. The background radioactivity, determined by omitting the DNA from the reaction mixture, was typically below 50 cpm, which is close to the background signal of the instrument. Data were corrected by subtraction of the background.

For the determination of K_M(DNA) and k_{cat}, the reaction mixtures (20 μL) contained Mtases (wild type, 0.38 nM; F196W, 1.14 nM; Y108W, 1.96 nM; Y108A and F196A, 11.4 nM), duplex ODN **8·9** (Y108A, 0.2−2 μM; wild type, 0.2−5 μM; F196A, 0.5−6.5 μM; Y108W, 0.83−6.5 μM; F196W, 1.0−12.0 μM), [methyl-^{3}H]-AdoMet (20 μM, mixed with a 9-fold excess of nonradioactive AdoMet, 1.5 Ci/mmol), and reaction buffer. Mtases and DNA in 1.1-fold concentrated reaction buffer (18 μL) were thermally equilibrated at 60 °C for 15 min, the reactions were started by adding [methyl-^{3}H]-AdoMet (2 μL), and aliquots (10 μL) were removed after 15 min. Separation of DNA and [methyl-^{3}H]-AdoMet and scintillation counting were done as described above. Rate constants (k) for each reaction were calculated from the obtained cpm values by subtracting the background radioactivity, obtained in a parallel experiment without DNA, and dividing through the reaction time, the specific radioactivity of [methyl-^{3}H]-AdoMet, and the molar amount of the Mtase.

For the determination of K_M(AdoMet) and k_{cat}, the reaction mixtures (20 μL) contained Mtases (F196W, 2.0 nM, wild type and Y108W, 4.0 nM; Y108A and F196A, 20 nM), duplex ODN **8·9** (10 μM), [methyl-^{3}H]-AdoMet (1, 1.25, 1.67, 2.5, 5.0, 10, and 20 μM, mixed with nonradioactive AdoMet to yield specific activities of 7.5, 6.0, 4.5, 3.0, 1.5, 0.75, and 0.375 Ci/mmol, respectively), and reaction buffer. Background radioactivity was determined by leaving out the DNA from the reactions containing 1 μM [methyl-^{3}H]-AdoMet. Reactions, separation of DNA and [methyl-^{3}H]-AdoMet, scintillation counting, and data processing were performed, as described above.

DNA Binding and Base Flipping Assay. Binding of mutant Mtases to the duplex ODN **10·12** was determined in solution at 25 °C, as described before for the wild-type enzyme (*17*). Briefly, **10·12** (250 nM in reaction buffer) containing the fluorescent 2-aminopurine base analogue at the target position was titrated with increasing amounts of the different mutant Mtases, and the 2-aminopurine fluorescence intensity was observed at an excitation wavelength of 320 nm and an emission wavelength of 381 nm. Fluorescence intensities were corrected for intrinsic protein fluorescence by subtraction of control titrations using the duplex ODN **11·12**. In addition, fluorescence data were corrected for varying background emission of the solutions. Titration data (fluorescence intensities as a function of total enzyme concentrations) were fitted to the real solution of the quadratic binding equation for one binding site (*32*) using the data analysis program GraFit (*33*).

RESULTS

Mutagenesis. Site-directed mutagenesis of the codons for Tyr 108 and Phe 196 of M·*Taq*I was achieved by two different methods. Replacement of the codon for Tyr 108 (TAC) by the codons for Phe (TTT) and Trp (TGG) was

performed using the in vitro mutagenesis procedure developed by Kunkel (*34*, *35*). Since the M·*Taq*I expression vector pAGL15-M13 carries the M13 origin of replication, it was possible to obtain single-stranded DNA containing uracil by growing this phagemid in an *Escherichia coli* strain, deficient in dUTPase and uracil-*N*-DNA-glycosylase activity, after infection with a helper phage. However, all attempts to mutagenise the *taqIM* gene directly within this expression vector were unsuccessful. Therefore, the *taqIM* gene was cloned as a *Nco*I/*Hind*III restriction fragment into a M13mp19 derivative carrying a *Nco*I and *Hind*III restriction site in the polylinker sequence. After successful mutagenesis, the mutated genes were subcloned into the expression vector and fully sequenced.

To mutagenise the *taqIM* gene directly in the expression vector, we used inverse PCR mutagenesis (*36*). The main advantage of the inverse PCR mutagenesis compared to different PCR mutagenesis methods is that no subcloning step is necessary and that only two primers in a single PCR are needed. The codon for Tyr 108 was randomly mutagenised using a degenerate plus strand primer containing all four bases at the three positions of the Tyr 108 codon and a regular minus strand primer. Amplification of the whole M·*Taq*I expression vector with a size of 5.6 kb was achieved by adding the thermostable *Pfu*-DNA-polymerase possessing a 3′ to 5′ exonuclease activity to the *Taq*-DNA-polymerase (*37*). From the obtained base substitution library, clones for the Gly (GGC) and for the Ala (GCC) replacements at codon 108 containing only the desired mutations in the Mtase gene were chosen for expression. In addition, inverse PCR mutagenesis was used to specifically replace the codon for Phe 196 (TTC) by the codons for the aromatic amino acid Trp (TGG) and the nonaromatic amino acid Ala (GCC). Two plasmids containing only the desired TGG or GCC codon at position 196 within the Mtase gene were used for expression.

The mutant proteins Y108W, Y108F, Y108A, Y108G, F196W, and F196A were expressed and purified to near homogeneity in analogy to the wild-type enzyme (*17*). As observed for the wild-type enzyme, most of the purified mutant Mtases contained bound AdoMet. The Mtase-bound cofactor was removed by incubation with a short palindromic ODN followed by anion-exchange chromatography as described before (*30*).

DNA Protection Assay. The catalytic properties of wild type and mutant proteins were first analyzed in a DNA protection assay, in which the biological function of M·*Taq*I is utilized (*38*). M·*Taq*I is part of the restriction−modification system from *Thermus aquaticus* and thus serves to protect the bacterial genome against the endogenous restriction endonuclease R·*Taq*I. In this assay λ-DNA is incubated with different amounts of M·*Taq*I or mutant proteins in the presence of saturating concentrations of AdoMet. Afterward an excess of R·*Taq*I is added, the incubation is continued, and the degree of protection is subsequently analyzed by agarose gel electrophoresis. The specific activities of wild-type and mutant enzymes were found to be 3200 (wild type), 1600 (Y108F), 200 (Y108W), 8 (Y108A), 1 (Y108G), 400 (F196W) and 8 units/μg (F196A). One unit is defined as the amount of Mtase required to fully protect one microgram of λ-DNA at 65 °C in 60 min against enzymatic cleavage by the R·*Taq*I restriction endonuclease. While the specific activities of Y108F, Y108W, and F196W carrying aromatic amino acid side chains at position 108 or 196 are only moderately reduced (2−16-fold) compared to that of the wild-type, the specific activities of Y108A, Y108G, and F196A having a nonaromatic side chain at these positions are reduced dramatically (400−3200-fold). This simple protection assay is suitable to obtain a rough estimate of the enzymatic activities of the different Mtases. However, it cannot be used to determine the catalytic constant k_{cat} and the Michaelis−Menten constant K_M, because this requires measurements of initial velocities. In addition, λ-DNA contains 121 M·*Taq*I recognition sites (5′-TCGA-3′), and both adenines within each palindromic recognition sequence need to be methylated in order to fully protect the DNA against cleavage by R·*Taq*I (unpublished observation). Thus, the DNA substrate in this simple protection assay is poorly defined.

Improved Radioactive Kinetic Assay. The mutants carrying the Trp and Ala residues at positions 108 and 196 as well as the wild-type enzyme were additionally analyzed with a defined hemimethylated duplex ODN in a kinetic assay, in which the rate of incorporation of the radioactive methyl group from [methyl-^{3}H]-AdoMet into DNA is measured. In this assay [methyl-^{3}H]-AdoMet, DNA, and Mtase are incubated, the DNA is separated from unreacted radioactive AdoMet, and the amount of radioactivity incorporated into the DNA substrate is measured by scintillation counting. The separation of DNA and unreacted radioactive AdoMet is typically done by transferring the reaction mixture onto a DEAE filter paper, which leads to binding of the DNA to the filter, and removing unreacted radioactive AdoMet by washing with low salt buffer. After the filters are dried, scintillation cocktail is added, and the radioactivity bound to the filters is measured by scintillation counting. Since tritium is a weak β-emitter, counting of tritiated compounds bound to solid supports normally gives rise to self-absorption quench, which can result in large errors (*39*). We, therefore, developed a method for counting the radioactive DNA in solution. The reaction mixture is applied to a small column filled with a DEAE-bound silica resin, and the radioactive AdoMet is washed away with low salt buffer. The DNA is then eluted with basic high salt buffer, and after addition of scintillation cocktail, the incorporated radioactivity is measured in solution with high accuracy. This method was performed in a micro titer plate format allowing a large number of samples to be handled simultaneously.

By using this improved radioactive assay for DNA Mtases, we first investigated the linearity of the assay and the thermostability of M·*Taq*I at 60 °C. We found that the radioactivity incorporated into the DNA increased linear over the observed time range of 25 min (Figure 2), demonstrating the thermostability of the wild-type enzyme. Corresponding results were obtained with the mutant proteins (not shown), which confirm that the thermostability of the mutant Mtases is not considerably affected by the mutations.

For the determination of the maximal turnover number k_{cat} and the Michaelis−Menten constants K_M for DNA and AdoMet, the reactions were performed at different substrate concentrations, and the radioactivity incorporated into the DNA was analyzed after 15 min. From the obtained velocities, the turnover numbers per active site (k) were calculated. Resulting Lineweaver−Burk plots (Figure 3)

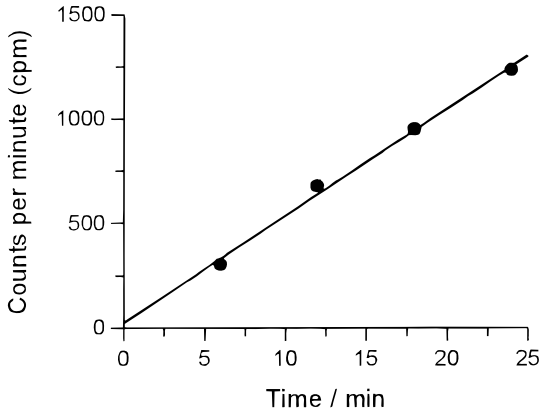


FIGURE 2: Linearity of the improved radioactive assay for DNA Mtases and thermostability of M·*Taq*I. Radioactivity (cpm) incorporated into the duplex ODN **8·9** by M·*Taq*I at 60 °C is plotted against the reaction time. Concentrations of [methyl-^{3}H]-AdoMet and DNA in the reaction mixture were 20 μM and 1.0 μM, respectively. Aliquots of the reaction mixture were withdrawn after different reaction times, and the DNA was separated from unreacted [methyl-^{3}H]-AdoMet by anion-exchange chromatography, allowing scintillation counting of the tritiated product DNA in solution.

showed a linear dependence of 1/k and 1/[DNA] or 1/[AdoMet], and the obtained k_{cat} and K_M values are listed in Table 1. The k_{cat} values of the mutant Mtases containing an aromatic Trp residue at position 108 or 196 are slightly reduced (Y108W) or even slightly increased (F196W) compared to k_{cat} of the wild-type enzyme. This is markedly different for the mutant Mtases having a nonaromatic Ala residue at these positions. The k_{cat} values of Y108A and F196A are reduced about 50- and 30-fold, respectively. In contrast to the k_{cat} values of the mutant Mtases, the K_M values for DNA and AdoMet show less variability. The K_M for DNA of Y108W and F196W compared to the K_M of the wild-type enzyme are increased to some extent, while those of Y108A and F196A are slightly reduced or unchanged, respectively. The K_M values for AdoMet of the mutants in comparison to the K_M value of the wild-type enzyme are more or less unchanged or slightly increased.

DNA Binding and Base Flipping Assay. The reason for the considerably reduced k_{cat} of the mutant Mtases Y108A and F196A was further investigated by DNA binding studies, using a duplex ODN in which the target adenine is replaced by the fluorescent base analogue 2-aminopurine. The 2-aminopurine fluorescence is highly quenched in polynucleotides due to interactions with neighboring bases and is strongly enhanced when the base is flipped out upon binding of the wild-type enzyme (*17*). Thus, fluorescence titrations with a duplex ODN containing 2-aminopurine at the target position can be used to determine dissociation constants K_D in solution. In addition, the magnitude of the fluorescence increase delivers information about the ability of the Mtase to flip out the target base. Fluorescence titrations of the duplex ODN containing 2-aminopurine with mutant and wild-type Mtases at 25 °C are shown in Figure 4. Obtained dissociation constants K_D and increases of the 2-aminopurine fluorescence intensity are listed in Table 2. The K_D values of the different Mtases for the duplex ODN containing 2-aminopurine show the same trend as their K_M values for DNA: K_D (Y108A) < K_D (wild type) < K_D (F196A) < K_D (Y108W) < K_D (F196W). Most interestingly, the fluorescence titrations yielded different 2-aminopurine fluorescence intensities of the various Mtase−DNA complexes.

DISCUSSION

Functional Role of Tyr 108. Tyr 108 of M·*Taq*I is part of the conserved motif IV (N/D/S P/I P Y/F/W-motif) found in N-DNA/RNA Mtases. Replacement of Tyr 108 by the aromatic amino acids Phe or Trp, which both match the consensus sequence of motif IV, leads to mutant Mtases with almost identical or somewhat reduced enzymatic activities, compared to the wild-type Mtase (see DNA protection assay). Especially the very similar enzymatic activities of the mutant Mtase Y108F and the wild-type enzyme demonstrate that the phenolic hydroxyl group of Tyr does not play an important role in catalysis by M·*Taq*I. In contrast to these aromatic amino acid replacements, the exchange of Tyr 108 for Ala or Gly results in mutant Mtases with dramatically reduced enzymatic activities which clearly demonstrate the importance of an aromatic amino acid side chain in motif IV for enzymatic activity. These results differ from those obtained with the N6-adenine DNA Mtases M·*Eco*P15I and M·*Eco*P1I, where the exchange of the corresponding Tyr 128 against Trp resulted in catalytically inactive proteins (*40*, *41*). However, our results are similar to those obtained with the N6-adenine DNA Mtase M·*Eco*KI (*42*). Replacement of the corresponding Phe 269 by Tyr and Trp in M·*Eco*KI resulted in mutant Mtases which possessed 25% and <5% of wild-type activity, respectively, while replacement by Ala resulted in an inactive mutant protein. However, no K_M values were reported for the active mutant Mtases. We, therefore, investigated M·*Taq*I and the mutant Mtases Y108W and Y108A in a steady-state kinetic assay which allows the determination of k_{cat} and K_M for DNA and AdoMet (Table 1). Compared to the wild type the mutant Mtase Y108W showed a slightly reduced k_{cat} (2-fold), a small increase in K_M for DNA (2.5-fold), and an almost unchanged K_M for AdoMet. The increase of K_M for DNA parallels a 2.5-fold higher K_D for DNA, as determined by fluorescence titrations with a duplex ODN containing 2-aminopurine at the target position (Table 2). The decreased k_{cat} and decreased affinity for DNA of Y108W suggest that DNA dissociation is not rate-limiting in the M·*Taq*I system. The absence of a burst phase in pre-steady-state experiments with the wild-type Mtase have in fact indicated that product release is not the rate-limiting step for catalysis by M·*Taq*I (*43*).[2] In this respect, M·*Taq*I behaves differently than the N-DNA Mtases M·*Eco*RI (*44*), M·*Mva*I (*45*), *Eco*Dam (*46*), and T4 Dam (*47*) which were shown to exhibit burst kinetics. Since DNA binding and base flipping by M·*Taq*I (*48*, *49*) are faster than the maximal reaction velocity, which was also demonstrated for the C5-cytosine DNA Mtase M·*Hha*I (*50*) and for the N6-adenine DNA Mtase M·*Eco*RI (*51*), k_{cat} in the M·*Taq*I

[2] Burst experiments were performed with duplex ODN **8·9** (15 μM), [methyl-^{3}H]-AdoMet (30 μM), and M·*Taq*I (60 nM) at 25, 37, and 60 °C. Reactions were rapidly quenched with perchloric acid (0.5 M final concentration) after different reactions times and aliquots analyzed as described for the steady-state assay. In addition, single-turnover experiments were performed. ODN **8·9** (1 μM), [methyl-^{3}H]-AdoMet (30 μM), and M·*Taq*I (1.1 μM) were incubated at 25 °C and reactions quenched and analyzed as in the burst experiments. Data were fitted to a single exponential function and revealed the same rate constant as that determined in the burst experiment at 25 °C demonstrating a high degree of active enzyme in the preparation and that the absence of a burst phase is not due to a partially active enzyme.

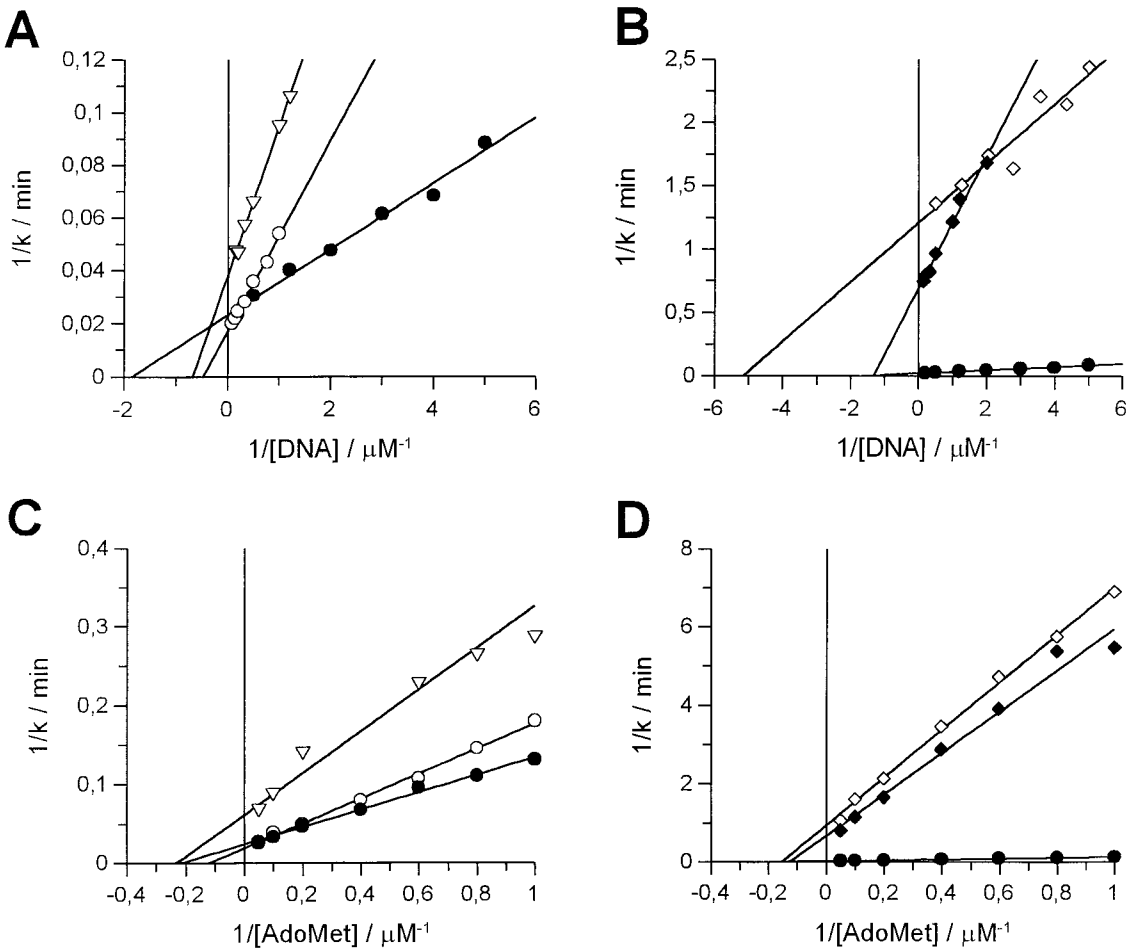


FIGURE 3: Linewever-Burk plots for the determination of k_{cat} and K_M for the duplex ODN **8·9** and for AdoMet of wild-type and mutant Mtases. To compare the different Mtases directly, we calculated rate constants (k) for each reaction from the obtained radioactivity incorporated into the duplex ODN (cpm values) using the specific radioactivity of [methyl-^{3}H]-AdoMet and the molar amount of Mtase. (A and B) Plots obtained with the wild-type Mtase (●) and the mutant Mtases Y108W (▽), F196W (○), Y108A (◇), and F196A (◆). The duplex ODN concentrations were varied around their K_M values, and the [methyl-^{3}H]-AdoMet concentration was kept constant at 20 μM. (C and D) Plots obtained with the wild-type Mtase (●) and the mutant Mtases Y108W (▽), F196W (○), Y108A (◇), and F196A (◆). The [methyl-^{3}H]-AdoMet concentrations were varied around their K_M values, and the concentration of duplex ODN **8·9** was kept constant at 10 μM.

Table 1: Maximal Turnover Number k_{cat} of Wild-Type and Mutant Mtases and Their Michaelis−Menten Constants K_M for DNA and AdoMet Determined in an Improved Radioactive Assay for DNA Mtases at 60 °C[a]

Mtase	k_{cat}[b] (min^{-1})	K_M (DNA)[b] (μM)	k_{cat}[c] (min^{-1})	K_M (AdoMet)[c] (μM)
wild type	44 ± 7	0.6 ± 0.2	43 ± 7	3.7 ± 1.1[d]
Y108W	26 ± 6	1.6 ± 0.7	17 ± 4	4.4 ± 1.6
Y108A	0.8 ± 0.2	0.2 ± 0.1	1.1 ± 0.2	6.5 ± 1.4
F196W	59 ± 4	2.2 ± 0.3	54 ± 7	8.6 ± 2.8
F196A	1.5 ± 0.1	0.8 ± 0.2	1.8 ± 0.3	10.1 ± 3.6

[a] Errors are given as 2σ standard deviations. [b] Determined at a constant [methyl-^{3}H]-AdoMet concentration of 20 μM and varying DNA concentrations. [c] Determined at a constant DNA concentration of 10 μM and varying [methyl-^{3}H]-AdoMet concentrations. [d] Mean value of two independent kinetic experiments.

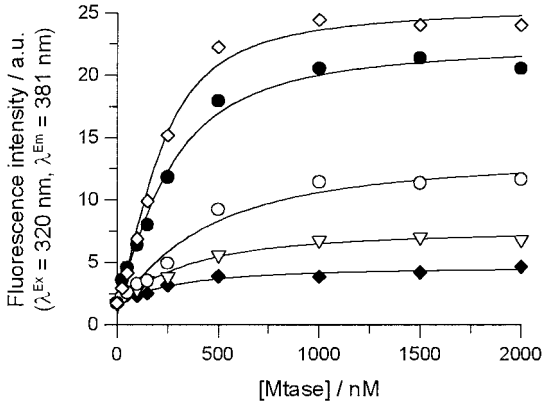


FIGURE 4: Fluorescence titrations of the duplex ODN **10·12** containing 2-aminopurine at the target position (250 nM) with wild-type Mtase (●) and the mutant Mtases Y108A (◇), F196W (○), Y108W (▽), and F196A (◆). Data for the wild-type Mtase are taken from ref 17.

system most likely reflects the kinetic barrier for methyl group transfer. However, k_{cat} may be modulated by the equilibrium between an extrahelical and innerhelical target adenine in the ternary complex, because only the fraction of ternary complex with an extrahelical target adenine is expected to be catalytically competent. The ability of the mutant Mtase Y108W for base flipping is proven by the increase of the 2-aminopurine fluorescence intensity in the fluorescence titration (Figure 4 and Table 2). Unfortunately, the 2-aminopurine fluorescence is quenched in hydrophobic environments (*52*), and the wild-type or mutant Mtases carrying amino acid side chains with varying hydrophobicities in the proposed active site may quench the fluorescence

Table 2: Dissociation Constants and Fluorescence Increases from Titrations of the Duplex ODN **10·12** Containing 2-Aminopurine at the Target Position with Wild-Type and Mutant Mtases[a]

Mtase	dissociation constant K_D (nM)	fluorescence increase (a.u.)
wild type[b]	120 ± 30	21.3 ± 1.3
Y108W	220 ± 60	6.0 ± 0.5
Y108A	70 ± 20	25.1 ± 1.4
F196W	290 ± 120	12.5 ± 1.6
F196A	200 ± 100	2.8 ± 0.4

[a] Fluorescence titrations were performed with a duplex ODN concentration of 250 nM at 25 °C, and the excitation and emission wavelengths were set to 320 and 381 nm, respectively. Fluorescence intensities are given in arbitrary units (a.u.) and errors as standard deviations. [b] Data from ref 17.

signal of the extrahelical 2-aminopurine to different extents. Thus, the reduced 2-aminopurine fluorescence intensity in the Y108W−DNA complex compared to the wild type−DNA complex could result at least partly from the more hydrophobic nature of Trp compared to Tyr residues (*53*).

The mutant Mtase carrying an Ala residue at position 108 (Y108A) showed a strongly reduced k_{cat} (50-fold), a small decrease in K_M for DNA (3-fold), and a slight increase in K_M for AdoMet (2-fold) compared to the wild-type enzyme. The decreased K_M for DNA is also reflected in a smaller K_D, as determined by fluorescence titration. Interestingly, the 2-aminopurine fluorescence intensity of the Y108A−DNA complex compared to the wild type−DNA complex is increased. This clearly indicates that Y108A is able to flip the target base, although some of the observed fluorescence intensity could be due to the exchange of Tyr for the less hydrophobic Ala, as discussed above. Thus, Tyr at position 108 in M·*Taq*I does not seem to stabilize the target base in an extrahelical conformation to a great extent. However, it does have a pronounced influence on the maximal turnover number k_{cat}. A possible role of Tyr at position 108 could be to place the extrahelical target base in an optimal position for methyl group transfer.

Functional Role of Phe 196. Phe 196 of M·*Taq*I forms the conserved motif VIII (F/Y/W) found in most N-DNA/RNA Mtases. Similar to the substitutions at position 108, replacing Phe 196 by the aromatic amino acid Trp resulted in a mutant Mtase with high enzymatic activity, whereas changing it to the nonaromatic amino acid Ala resulted in a mutant enzyme with strongly reduced enzymatic activity (see DNA protection assay). This clearly demonstrates the importance of an aromatic amino acid in motif VIII for enzymatic activity. Compared to the wild-type enzyme, k_{cat}, K_M for DNA, and K_M for AdoMet of the mutant Mtase F196W are somewhat increased (1.3-, 3.5-, and 2-fold, respectively; Table 1). A 2.5-fold increase in K_D for DNA is seen in the fluorescence binding assay. The fluorescence intensity of the F196W−DNA complex is about 2-fold smaller compared to the wild-type Mtase, which could be explained by the more hydrophobic nature of Trp compared to Phe (*53*), leading to a stronger quenching of the extrahelical 2-aminopurine base. Thus, the mutant Mtases F196W as well as Y108W behave quite similarly compared with the wild-type enzyme.

Replacement of Phe 196 with Ala leads to a mutant Mtase with a 30-fold decreased k_{cat}, an almost unchanged K_M for DNA, and a modestly increased K_M for AdoMet. The most interesting result for this mutant Mtase was obtained in the fluorescence titration, which showed only a very small increase of the 2-aminopurine fluorescence. Since Ala is less hydrophobic than Phe, the much smaller fluorescence intensity of the F196A−DNA complex compared to the wild type-DNA complex cannot be explained by a more pronounced quenching of an extrahelical 2-aminopurine. This result demonstrates that the mutant Mtase F196A is strongly impaired in its ability to stabilize the target base in its extrahelical position and that the equilibrium between the extrahelical and innerhelical target base is shifted toward the innerhelical side. Since only the fraction of ternary complex with an extrahelical target adenine should be catalytically competent, at least some of the reduction in k_{cat} can be readily explained. This result also strongly indicates that Phe at position 196 plays an important role in stabilizing the extrahelical target adenine, which could be achieved by aromatic π-interactions.

Since the influence of amino acid replacements at position 108 and 196 on K_M for AdoMet is rather small, Tyr 108 and Phe 196 do not seem to play an important role in AdoMet binding. This is in complete agreement with the structure of M·*Taq*I in complex with the cofactor AdoMet, where no direct contacts between Tyr 108 or Phe 196 and AdoMet are found (Figure 1, refs 18, 30).

In conclusion, our results from a mutational analysis of Tyr 108 and Phe 196 in M·*Taq*I are consistent with a structural model, in which the extrahelical adenine interacts with these aromatic amino acids (*27*). Our data indicate that Phe 196 is involved in the stabilization of the extrahelical target base, while this function is much less pronounced for Tyr 108. Tyr 108 probably helps to place the extrahelical target base in an optimal position for methyl group transfer. In addition, our data are in accordance with a stabilization of a positively charged intermediate (transition state) by cation-π interactions with these two aromatic residues (*27*). However, this functional role is not certain and requires further investigations. Since both aromatic amino acids belong to the conserved motif IV (N/D/S P/I P Y/F/W) and motif VIII (F/Y/W) found in N-DNA/RNA Mtases, a similar function of aromatic amino acids within these motifs is expected for other Mtases.

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