DNA methyltransferases of the Dam family (including bacteriophage T4-encoded Dam DNA (adenine-$N^6$)-methyltransferase (T4Dam)) catalyze methyl group transfer from S-adenosyl-L-methionine (AdoMet), producing S-adenosyl-L-homocysteine (AdoHcy) and methylated adenine residues in palindromic GATC sequences. In this study, we describe the application of direct (i.e. no exogenous cross-linking reagents) laser UV cross-linking as a universal non-perturbing approach for studying the characteristics of T4Dam binding with substrates in the equilibrium and transient modes of interaction. UV irradiation of the enzyme-substrate complexes using an Nd$^{3+}$:yttrium aluminum garnet laser at 266 nm resulted in up to 3 and $>15\%$ yields of direct T4Dam cross-linking to DNA and AdoMet, respectively. Consequently, we were able to measure equilibrium constants and dissociation rates for enzyme-substrate complexes. In particular, we demonstrate that both reaction substrates, specific DNA and AdoMet (or product AdoHcy), stabilized the ternary complex. The improved substrate affinity for the enzyme in the ternary complex significantly reduced dissociation rates (up to 2 orders of magnitude). Several of the parameters obtained (such as dissociation rate constants for the binary T4Dam:AdoMet complex and for enzyme complexes with a non-fluorescent hemimethylated DNA duplex) were previously inaccessible by other means. However, where possible, the results of laser UV cross-linking were compared with those of fluorescence analysis. Our study suggests that rapid laser UV cross-linking efficiently complements standard DNA methyltransferase-related tools and is a method of choice to probe enzyme-substrate interactions in cases in which data cannot be acquired by other means.

The important epigenetic process of DNA methylation has been found in members of virtually every major biological group, from viruses to mammals. This reaction is carried out by DNA methyltransferases (MTases), which catalyze methyl group transfer from the donor S-adenosyl-l-methionine (AdoMet), producing S-adenosyl-l-homocysteine (AdoHcy) and methylated cytosine or adenine bases within specific recognition sites. The common role of MTases in prokaryotes and their phages is to protect DNA from the cell’s own restriction enzymes. However, some MTases are known to act as regulators of gene expression and affect other critical functions (1, 2). The most widespread among the latter are the homologous Dam MTases, which methylate an exocyclic $N^6$-amino group of adenine in palindromic 5'-GATC sites (3). It should be noted that Dam methylation interferes with the coordinated expression of virulence factors in an increasing number of pathogenic bacterial species, including the Yersinia and Vibrio genera (4). Consequently, a detailed physical-chemical and structural characterization of Dam enzymes is clearly of great importance because it could spur development of a new class of antibiotics based on their ability to regulate Dam activity (5).

The subject of this study is the bacteriophage T4-encoded Dam DNA (adenine-$N^6$)-methyltransferase (T4Dam) (6, 7), which belongs to a large family of homologous Dam enzymes. Amino acid sequence homology between proteins may indicate that a considerable degree of similarity exists in their ternary structures and in their mechanisms of action. Taking into account the array of available x-ray structural data, including a binary enzyme-AdoHcy complex and six structures of enzyme-DNA complexes in non-, semi-, and fully specific modes of their interaction along the DNA recognition pathway (8, 9), T4Dam provides an excellent model system. During an earlier physical-chemical study of this enzyme, we applied conventional electrophoretic mobility shift and 2-amino-purine-based binding assays as well as steady-state and pre-steady-state kinetic assays to characterize T4Dam-catalyzed methylation using a wide range of oligodeoxynucleotide substrate duplexes (10–14). In particular, our observations indicated that AdoMet has a function in addition to its serving as the methyl donor, viz. T4Dam alone binds randomly to a hemi-methylated palindromic site, whereas the addition of AdoMet
Bacteriophage T4 Dam DNA (Adenine-N⁶)-methyltransferase

induces a reorientation of the enzyme to the strand containing the unmethylated target base (13). Another important observation is that methylation of DNAs having more than one specific site is affected by the enzyme’s processivity (14), i.e. its ability to undergo facilitated linear diffusion along the DNA and to carry out multiple turnovers on the same substrate molecule. Such a processive methylation was also shown for another homologous enzyme, the Dam MTase of Escherichia coli (15). Apparently, both these mechanisms strongly increase the efficiency of methylation of hemimethylated DNA produced by in vivo replication.

One of the unanswered questions regarding the mechanism of the T4Dam-catalyzed reaction concerns the dynamics of specific DNA and cofactor binding and their reciprocal influence on the affinity for the enzyme in the ternary complex. In this study, we describe the application of a direct (i.e. no exogenous cross-linking reagents) laser-mediated UV cross-linking technique as a universal non-perturbing approach to investigate the characteristics of T4Dam binding with its substrates in the equilibrium and transient modes of their interaction. For this purpose, we used both conventional equilibrium and novel time-resolved laser UV cross-linking based on the well known ability of laser UV irradiation to induce photochemical cross-linking of proteins with specific nucleic acids (16–18) and on the fact that prolonged low energy UV irradiation can “fix” MTase-AdoMet complexes (19–25).

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—The T4Dam MTase was purified to homogeneity as described (26). The protein concentration was determined by the Bradford method (27), which yielded values in close agreement with those measured spectrophotometrically at 280 nm from the known composition and molar extinction coefficients of individual aromatic amino acid residues (ε₂₈₀ = 34990 M⁻¹ cm⁻¹) in 6.0 M guanidine hydrochloride and 0.02 M phosphate buffer (pH 6.5) (28). [methyl-³²H]AdoMet (15 Ci/mmol) and [γ-³²P]ATP (3 KCi/mol) were from Amersham Biosciences. Unlabeled AdoMet (purified further by reversed-phase chromatography) (26), AdoHcy, and sinefungin were from Sigma. High pressure liquid chromatography-purified 20-mer oligodeoxynucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX); the concentrations were determined spectrophotometrically. The following specific substrate duplexes were obtained by annealing complementary oligodeoxynucleotide chains (where M is N⁶-methyladenine and N is 2-aminopurine): 5’-CAGTTTGGATCCATTCAC-3’ and 3’-GTCAAATCTTAGGTAAAGTG-5’ (A/A); 5’-CAGTTTGGATTCATTCAC-3’ and 3’-GTCAAATCTTAGGTAAAGTG-5’ (M/A); 5’-CAGTTTCAGTTCATTCAC-3’ and 3’-GTCAAATCTTAGGTAAAGTG-5’ (M/M); and 5’-CAGTTTGGATTCATTCAC-3’ and 3’-GTCAAATCTAGGTAAAGTG-5’ (M/N). All experiments were carried out at 25 °C in standard T4Dam reaction buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 0.2 mg/ml bovine serum albumin (26).

Laser UV Cross-linking of MTase with DNA—Equilibrium T4Dam-DNA cross-linking was performed in 20 µl of T4Dam reaction buffer containing 7 nM ³²P-labeled specific oligodeoxynucleotide duplex, 2–60 nM T4Dam, and, in some experiments, 20 µM AdoHcy or AdoMet. Following a 5-min incubation, a single 5-ns laser pulse of 266 nm irradiation with the fourth harmonic of an Nd³⁺:yttrium aluminum garnet laser (DCR-3G, Spectra-Physics) was applied to the sample. An optical system consisting of two frequency-doubling crystals and a series of mirrors allowed an incident beam at 266 nm to be vertically focused on the sample placed in an unsealed 0.5-ml Eppendorf tube. The laser worked in repetitive pulsed mode, generating 10 pulses/s; single-pulse irradiation was achieved using an electromagnetic shutter. Under optimum conditions, the irradiation dose was ~40 mJ (10¹⁷ photons)/pulse, sufficient for saturation of T4Dam-DNA adduct formation (up to 3% of the protein-DNA adduct). After irradiation, samples were diluted with SDS-PAGE loading buffer, heated at 95 °C for 5 min to ensure protein denaturation, and subjected to 12% SDS-PAGE. Unirradiated mixtures were used as negative controls. Gels were dried and analyzed by phosphorimaging densitometry. Quantification was carried out using ImageQuant 5.0 software (GE Healthcare). The results of densitometry were confirmed by liquid scintillation counting of ³²P radioactivity in excised gel slices corresponding to the separate bands.

In time-resolved dissociation experiments, a microvolume quench-flow RQF-3 instrument (KinTek Corp.) was included in the experimental setup. A 15-µl aliquot of a preformed T4Dam-[³²P]DNA or T4Dam-AdoHcy-[³²P]DNA complex (12 nM complex in the final 30-µl mixture) in one syringe was rapidly mixed with an equal volume of unlabeled DNA (100-fold excess) in another syringe. The mixture was incubated in the reaction loop during an appropriate time interval and injected into the quartz flow cell, followed by a single pulse of laser irradiation linked to the Q-switch emission of the laser. The resulting dead time of our experiments was on the order of ~50 ms.

Laser UV Cross-linking of MTase with AdoMet—The experimental procedure above permitted us to also perform T4Dam-AdoMet cross-linking. In this case, adduct formation was hyperbolically dependent on the dose of irradiation. The reaction mixture (8-µl final volume) for equilibrium T4Dam-AdoMet cross-linking contained 0.4 µM T4Dam, 0.1–10 µM [methyl-³²H]AdoMet, and, in one experiment, 0.5 µM duplex M/N (with N⁶-methyladenine and 2-aminopurine in the target positions of the GATC site; not a substrate for methyl transfer). Following a 5-min preincubation, the sample was irradiated using the laser. Unirradiated mixtures were used for background corrections at each AdoMet concentration. Following irradiation, samples were denatured by heating at 95 °C for 5 min with 0.1% SDS and spotted onto nitrocellulose membrane filters (0.45 µm; Bio-Rad). Filters were washed three times to remove free AdoMet with phosphate-buffered buffer and once with water and then dried. The amount of [methyl-³²H]AdoMet radioactivity cross-linked to protein was quantified by liquid scintillation counting. To obtain a high enough signal-to-background ratio (~10) under variable background conditions, a series of 10 laser pulses was applied to the samples during these equilibrium titrations.
In the time-resolved dissociation experiments, a 15-μl aliquot of preformed T4Dam-[methyl-3H]AdoMet or T4Dam-[methyl-3H]-AdoMet-M/N complex (1 μM T4Dam, 1 μM [methyl-3H]AdoMet, and 1.2 μM duplex M/N in the 30-μl final mixture) was rapidly mixed and incubated for an appropriate interval with an equal volume of unlabeled AdoMet (20-fold excess), followed by laser irradiation. Under these conditions, a single pulse was applied to the sample. It should be noted that the use of much higher total concentration of ligands, especially unlabeled ones, may lead to poor adduct formation because of the high intrinsic absorption of UV light (inner filter effect) and hence quenching of the reaction by these ligands.

Equilibrium Fluorescence Analysis of MTase-Cofactor Interactions—Fluorescence intensities from equilibrium titrations were measured on a dual-beam difference Shimadzu RF-520 spectrofluorometer. T4Dam alone (1.0 μM) was titrated by incremental addition of AdoMet (2.5–55 μM) in reaction buffer with no albumin. In this case, the intrinsic Trp fluorescence of the protein was excited and detected at wavelengths of 295 nm (slit width of 5 nm) and 340 nm (slit width of 10 nm), respectively. A second control cuvette lacking AdoMet was included to correct for a strong photobleaching effect. In another experiment, T4Dam (160 nM) complexed with 2-aminopurine-substituted non-reactive duplex M/N (160 nM) was titrated with AdoMet (0.25–20 μM). The fluorescence was excited and detected at wavelengths of 320 nm (slit width of 5 nm) and 370 nm (slit width of 10 nm), which correspond to the maxima of 2-aminopurine excitation and emission, respectively (13).

2-Aminopurine-based Stopped-flow Studies—Stopped-flow experiments were performed on an Applied Photophysics SX.18MV spectrometer equipped with a 150-watt xenon arc lamp and a 2-mm path length optical cell. Fluorescence emission from 2-aminopurine-substituted non-reactive duplex M/N was observed using a 360-nm long-pass filter. The excitation wavelength was 320 nm with a 14-nm monochromator slit. The dead time of the stopped-flow instrument was 1.4 ms. The preincubated T4Dam-M/N or T4Dam-M/N-AdoMet complex (160 nM T4Dam, 250 nM duplex M/N, and 5 μM AdoMet) was rapidly mixed with an equal volume of either concurrent non-fluorescent duplex A/A (50-fold excess) or AdoMet at varied concentrations (in the case of the T4Dam-M/N complex). Typically, 5–10 fluorescence traces (1000 data points each collected logarithmically with time) were averaged and analyzed for each experiment.

Data Analysis—Numerical data were analyzed using Origin 6.1 software (OriginLab). Equilibrium curves (optical densities, counts/min values, or fluorescence intensities) were fitted to a quadratic binding equation (Equation 1) that takes into account ligand depletion (29). A representative autoradiograph and the resulting titration curves are shown in Fig. 1, in which the intensity of the cross-linked adducts can be seen to increase as a function of protein concentration.

The salient findings are summarized as follows (Table 1). (i) The affinity of T4Dam for DNA containing a non-methylated (A/A), hemimethylated (M/A), or fully methylated (M/M) site was M/A > M/M > A/A. (ii) In the ternary complex with AdoHcy, the relative affinity was M/A > M/M ≈ A/A. Bound AdoHcy led to 8- and 3.5-fold higher affinities of the enzyme for duplexes M/A and A/A, respectively, and did not appreciably change the apparent $K_d$ for the methylated DNA product M/M. However, the enzyme’s affinity for duplex M/M was 2-fold lower in the presence of AdoMet. These results are in agreement with those reported for other MTases. Thus, AdoMet and its non-reactive analogs generally improve the affinity of an MTase for its substrate DNA. For instance, AdoHcy induced a thousandfold tighter binding of the specific 20-mer duplex with a GTAC palindrome in place of 2-aminopurine-basedStopped-flow studies showed good yields of direct cross-linking (up to 3%) (16). A single laser pulse (~40 ml) was sufficient to saturate adduct formation. The cross-linked product remained stable after 5 min of incubation with SDS at 95 °C. The specificity of the cross-link was confirmed using a non-specific 20-mer duplex with a GTAC palindrome in place of GATC, in which case weak adduct formation was observed only at micromolar concentrations of ligands (data not shown). To probe further the capabilities of the laser UV cross-linking technique, we carried out a series of equilibrium T4Dam-DNA binding experiments. To prevent methylation of non- and hemimethylated substrate duplexes, we used the methyl donor product AdoHcy instead of AdoMet in our titrations. Because the concentrations of DNA and enzyme were comparable, equilibrium binding curves were fitted to Equation 1, which takes into account ligand depletion (29). A representative autoradiograph and the resulting titration curves are shown in Fig. 1, in which the intensity of the cross-linked adducts can be seen to increase as a function of protein concentration.

The salient findings are summarized as follows (Table 1). (i) The affinity of T4Dam for DNA containing a non-methylated (A/A), hemimethylated (M/A), or fully methylated (M/M) site was M/A > M/M > A/A. (ii) In the ternary complex with AdoHcy, the relative affinity was M/A > M/M ≈ A/A. Bound AdoHcy led to 8- and 3.5-fold higher affinities of the enzyme for duplexes M/A and A/A, respectively, and did not appreciably change the apparent $K_d$ for the methylated DNA product M/M. However, the enzyme’s affinity for duplex M/M was 2-fold lower in the presence of AdoMet. These results are in agreement with those reported for other MTases. Thus, AdoMet and its non-reactive analogs generally improve the affinity of an MTase for its substrate DNA. For instance, AdoHcy induced a thousandfold tighter binding of the specific hemimethylated duplex to the (cytosine-C5)-MTase HhaI (30, 31). The data on the relative affinity in the binary and ternary complexes of T4Dam with DNA duplexes having different states of methylation are consistent with previous reports for the RsrI (32) and EcoRV (33) (adenine-N6)-MTases. (iii) The laser UV cross-linking assay gave lower values for equilibrium binding constants compared with previously reported results from electrophoretic mobility shift assay (10) for the T4Dam-A/A (27 versus 43 nM) and T4Dam-M/A (4.2 versus 23 nM) complexes (Table 1). The gel shift assay is documented as
not being an optimum method for probing equilibrium protein-DNA interactions because of cage effects experienced as molecules enter the gel and because of dissociation of the complex as it passes through the gel. Therefore, we suggest that the laser UV cross-linking gave more accurate \( K_d \) values because of the short time of the laser irradiation pulse (5 ns in our case) and the ensuing photochemistry that covalently freezes an instant “snapshot” of the enzyme-substrate complex population. However, it should be noted that the sensitivity of direct laser cross-linking is lower compared with the gel shift assay, being limited by the cross-linking yield. On the other hand, the alternative non-radioactive fluorescence DNA binding assays (such as 2-aminopurine- or polarization anisotropy-based) are less sensitive and fail to directly measure equilibrium constants around and below 10 nM, as is the case for T4Dam MTase.

**Equilibrium Binding of T4Dam to AdoMet**—In preliminary experiments, we used increasing doses of irradiation (1–100 laser pulses) at 5 \( \mu \text{M} \) enzyme and 10 \( \mu \text{M} \) [methyl-\(^3\)H]AdoMet to estimate the yield of T4Dam-AdoMet adduct formation. The yield of cross-linking was hyperbolically dependent on the dose of irradiation; half-saturation of T4Dam-AdoMet adduct formation kinetics was observed after four to five irradiation pulses (Fig. 2A). After background subtraction, the amounts of cross-linked AdoMet were normalized to the concentration of the enzyme. We obtained up to 15% cross-linking; this value is a lower limit, taking into account the loss of adduct caused by the filter washing procedure and incomplete saturation of the enzyme with [methyl-\(^3\)H]AdoMet (10 \( \mu \text{M} \) versus \( K_d = 5.7 \mu \text{M} \); see below). The cross-linked adduct was stable after 5 min of incubation in SDS at 95 °C. T4Dam-[methyl-\(^3\)H]AdoMet cross-linking was inhibited by the presence of unlabeled AdoMet, AdoHcy, or sinefungin (adenosylornithine); the latter two compounds are non-reactive analogs of AdoMet and competitive inhibitors of the methylation reaction (34). It was interesting to compare T4Dam-AdoMet equilibrium binding constants in the binary complex versus the ternary complex with specific DNA. To prevent the methylation reaction, duplex M/N was used as a non-reactive analog of hemimethylated substrate duplex M/A. Titration experiments were performed by varying the [methyl-\(^3\)H]AdoMet concentration, and the data were fitted to Equation 1. We observed that

**TABLE 1**

**Equilibrium T4Dam-substrate binding constants**

| Substrate                  | \( K_d \) (nm) | Alternative assay |
|----------------------------|----------------|------------------|
| A/A                       | 4.2 ± 0.8      | 23\(^{a}\)       |
| M/A supplemented with AdoHcy | 0.53 ± 0.30    |                  |
| A/A                       | 27 ± 7         | 43\(^{a}\)       |
| M/M supplemented with AdoHcy | 7.6 ± 1.3      |                  |
| M/M                       | 7.2 ± 0.9      |                  |
| M/M supplemented with AdoMet | 7.3 ± 1.2    |                  |
| M/M supplemented with M/N | 16 ± 4         |                  |
| AdoMet                    | 5700 ± 600    | 9300 ± 4900\(^{b}\) |
| AdoMet supplemented with M/N | 450 ± 40     | 302 ± 54\(^{c}\) |

\(^{a}\) Data from Ref. 10 were obtained by gel shift assay.

\(^{b}\) The value was obtained by equilibrium quenching of the intrinsic fluorescence of enzyme Trp residues with AdoMet in the binary complex (Fig. 2C).

\(^{c}\) The value was obtained by equilibrium quenching of the fluorescence of duplex M/N with AdoMet in the ternary complex (Fig. 2D).
the addition of specific DNA led to 12-fold tighter enzyme-AdoMet binding (Fig. 2B and Table 1). It should be noted that, to increase the signal-to-background ratio, we applied a series of 10 laser pulses to the samples during equilibrium titrations with [methyl-3H]AdoMet. This introduces some uncertainty into determination of the binding constant because of possible photochemical destruction of ligands under repetitive laser irradiation, which may cause an increase in the apparent $K_D$. Apparently, the simple nitrocellulose filter binding assay used here may not be an optimum procedure for detection of MTase-AdoMet cross-linked adducts because of the high background from free unwashed [methyl-3H]AdoMet. We suggest that an improved protein binding assay or another method of adduct detection (such as SDS-PAGE, followed by fluorography) might allow the use of single-pulse laser irradiation over a wide range of [methyl-3H]AdoMet concentrations. Another way to increase cross-linking yields under single-pulse irradiation might be to use higher irradiation energies.

Nevertheless, the constant obtained for the binary T4Dam-AdoMet complex (5.7 μM) was within the range of values (2–11 μM) reported for other MTases (19, 22, 25, 30–32, 35–37), and the constant for the ternary T4Dam-AdoMet-DNA complex was close to the steady-state Michaelis constant for AdoMet ($K_m = 0.45$ versus 0.49 μM) (11). Moreover, these results agreed reasonably with independent measurements performed by the fluorescence methods. The equilibrium AdoMet binding with T4Dam alone was tested using known quenching of intrinsic enzyme Trp fluorescence in the binary T4Dam-cofactor complex (38) at a wide range of cofactor concentrations (up to 55 μM). It should be noted that, although significant (~35%) fluorescence quenching was observed (Fig. 2C), the amplitude of the signal was rather small and was subjected to a strong time-dependent photobleaching. In our experience, these observations preclude corresponding stopped-flow Trp fluorescence quenching studies. Therefore, the equilibrium binding constant was determined with a relatively large error (9.3 ± 4.9 μM) (Table 1). The binding of AdoMet to the preformed T4Dam-DNA complex was measured using (as described above) duplex M/N, bearing in mind the known quenching of 2-aminopurine (N) fluorescence in the ternary complex with a cofactor (13). As shown in Fig. 2D, the addition of specific DNA expectedly led to a sharp increase in AdoMet affinity for the enzyme in the ternary complex (302 nM) (Table 1).

**Kinetics of T4Dam-Substrate Binding**—Given that the quantal yield is constant for a single dose of laser irradiation, this allowed time-resolved analysis of enzyme-substrate binding. However, because of the relatively long dead time of our experimental setup (~50 ms), we restricted these studies to the measurement of slower dissociation (compared with association) rates. Initially, we measured the rate constants of the dissociation of canonical hemimethylated substrate duplex M/A from binary and ternary complexes. The data were fitted to Equation 2, and using equilibrium parameters as described above, the corresponding association rate constants were calculated (Fig. 3, A and B; and Table 2). The measured dissociation rate constants of the binary T4Dam-AdoMet complex are consistent with those obtained for other MTases (19, 22, 25, 30–32, 35–37), and the constant for the ternary T4Dam-AdoMet-DNA complex was close to the steady-state Michaelis constant for AdoMet ($K_m = 0.45$ versus 0.49 μM) (11). Moreover, these results agreed reasonably with independent measurements performed by the fluorescence methods. The equilibrium AdoMet binding with T4Dam alone was tested using known quenching of intrinsic enzyme Trp fluorescence in the binary T4Dam-cofactor complex (38) at a wide range of cofactor concentrations (up to 55 μM). It should be noted that, although significant (~35%) fluorescence quenching was observed (Fig. 2C), the amplitude of the signal was rather small and was subjected to a strong time-dependent photobleaching. In our experience, these observations preclude corresponding stopped-flow Trp fluorescence quenching studies. Therefore, the equilibrium binding constant was determined with a relatively large error (9.3 ± 4.9 μM) (Table 1). The binding of AdoMet to the preformed T4Dam-DNA complex was measured using (as described above) duplex M/N, bearing in mind the known quenching of 2-aminopurine (N) fluorescence in the ternary complex with a cofactor (13). As shown in Fig. 2D, the addition of specific DNA expectedly led to a sharp increase in AdoMet affinity for the enzyme in the ternary complex (302 nM) (Table 1).

**Kinetics of T4Dam-Substrate Binding**—Given that the quantal yield is constant for a single dose of laser irradiation, this allowed time-resolved analysis of enzyme-substrate binding. However, because of the relatively long dead time of our experimental setup (~50 ms), we restricted these studies to the measurement of slower dissociation (compared with association) rates. Initially, we measured the rate constants of the dissociation of canonical hemimethylated substrate duplex M/A from binary and ternary complexes. The data were fitted to Equation 2, and using equilibrium parameters as described above, the corresponding association rate constants were calculated (Fig. 3, A and B; and Table 2). The measured dissociation rate constants of the binary T4Dam-AdoMet complex are consistent with those obtained for other MTases (19, 22, 25, 30–32, 35–37), and the constant for the ternary T4Dam-AdoMet-DNA complex was close to the steady-state Michaelis constant for AdoMet ($K_m = 0.45$ versus 0.49 μM) (11). Moreover, these results agreed reasonably with independent measurements performed by the fluorescence methods. The equilibrium AdoMet binding with T4Dam alone was tested using known quenching of intrinsic enzyme Trp fluorescence in the binary T4Dam-cofactor complex (38) at a wide range of cofactor concentrations (up to 55 μM). It should be noted that, although significant (~35%) fluorescence quenching was observed (Fig. 2C), the amplitude of the signal was rather small and was subjected to a strong time-dependent photobleaching. In our experience, these observations preclude corresponding stopped-flow Trp fluorescence quenching studies. Therefore, the equilibrium binding constant was determined with a relatively large error (9.3 ± 4.9 μM) (Table 1). The binding of AdoMet to the preformed T4Dam-DNA complex was measured using (as described above) duplex M/N, bearing in mind the known quenching of 2-aminopurine (N) fluorescence in the ternary complex with a cofactor (13). As shown in Fig. 2D, the addition of specific DNA expectedly led to a sharp increase in AdoMet affinity for the enzyme in the ternary complex (302 nM) (Table 1).
Bacteriophage T4 Dam DNA (Adenine-N⁶)-methyltransferase

FIGURE 3. Time course of dissociation of T4Dam-substrate complexes measured by rapid laser UV cross-linking. A and B, cross-linking of enzyme with [³²P]-labeled duplex M/A. A preformed T4Dam-DNA or T4Dam-AdoHcy-DNA complex (12 nM final concentration) was rapidly mixed and incubated during an appropriate interval with an equal volume of unlabeled duplex M/A (100-fold excess). This was followed by a single pulse of irradiation as described under "Experimental Procedures." After protein denaturation with SDS, the cross-linked adduct was separated from free DNA by 12% SDS-PAGE and quantified by phosphorimaging densitometry. A, representative autoradiograph of PAGE analysis of T4Dam-M/A adduct dissociation constant of the T4Dam-M/A complex (0.50 s⁻¹) was 40-fold slower in the presence of AdoHcy (0.013 s⁻¹). The latter value is close to the steady-state catalytic turnover constant for the A/A-to-M/A conversion reaction (0.015 s⁻¹), which is rate-limited by T4Dam-M/A:AdoHcy product complex dissociation (11). The calculated rate constants for enzyme-DNA association (120 μM⁻¹ s⁻¹ for the binary complex and 25 μM⁻¹ s⁻¹ for the ternary complex) are close to the diffusion-controlled limit (~1000 μM⁻¹ s⁻¹) (39) and lie within the range of values measured by stopped-flow assays for the HhaI (1300 μM⁻¹ s⁻¹) (40), *E. coli* Dam (75 μM⁻¹ s⁻¹) (41), EcoRI (11–22.5 μM⁻¹ s⁻¹) (42), and EcoRV (7.0 μM⁻¹ s⁻¹) (43) MTases.

Using the same experimental setup, we also measured the rate constants of dissociation of AdoMet from the binary and ternary complexes with enzyme and then calculated the corresponding association rate constants (Fig. 3C and Table 2). The constants for the binary T4Dam:AdoMet complex association/dissociation (k_on = 1.3 μM⁻¹ s⁻¹ and k_off = 7.4 s⁻¹) are close to those reported for the (cytosine-C⁵)-MTase HhaI (k_on = 3.54 μM⁻¹ s⁻¹ and k_off = 21.8 s⁻¹) (31); as in the case of release of DNA, the apparent dissociation rate constant for the ternary complex in the presence of specific DNA (0.14 s⁻¹) was significantly lower (50-fold) compared with that for the binary complex (7.4 s⁻¹). Using the latter value, we estimated the overall goodness of fit for the transient parameter determination. According to the thermodynamic constraint for the cycle of ternary complex formation, the product of four rate constants in a clockwise sense must be equal to the product of the four counterclockwise constants. Consequently, we can write \( \frac{k_{on}(\text{DNA}(\text{binary}),\text{AdoMet}(\text{ternary}),\text{DNA}(\text{ternary}),\text{AdoMet}(\text{binary}))}{k_{off}(\text{AdoMet}(\text{binary}),\text{DNA}(\text{ternary}),\text{AdoMet}(\text{ternary}),\text{DNA}(\text{binary}))} = 1 \).

The substitution of values from Table 2 into this equation gives k_on/m_A = 0.20 (0.17 × 0.11). The relative optical densities (OD rel.) for cross-linked adducts for T4Dam complexed with duplex M/A alone (●) or in the presence of 20 μM AdoHcy (■), cross-linking of the T4Dam[¹⁴C]-AdoMet complex alone (●) or in the presence of specific non-reactive duplex M/N (■), is shown. A preformed T4Dam-AdoMet or T4Dam-AdoMet/M/N complex (1 μM T4Dam, 1 μM [¹⁴C]-AdoMet, and 1.2 μM duplex M/N in the final mixtures) was rapidly mixed and incubated for an appropriate time interval with an equal volume of unlabeled AdoMet (20-fold excess), followed by a single pulse of laser irradiation. Data were fitted to Equation 2 to give apparent dissociation rate constants as shown in Table 2.

### TABLE 2
Rate constants of substrate dissociation from the complex with T4Dam measured by the rapid laser UV cross-linking and the corresponding calculated association rate constants

| Substrate | k_on \( s^{-1} \) | k_on \( \mu M^{-1} s^{-1} \) | k_off \( s^{-1} \) | k_off \( \mu M^{-1} s^{-1} \) |
|-----------|----------------|-----------------|-----------------|-----------------|
| M/A       | 0.50 ± 0.08    | 120             |                 |                 |
| M/A supplemented | 0.013 ± 0.003 | 25              |                 |                 |
| AdoMet    | 7.4 ± 2.9      | 1.3             |                 |                 |
| AdoMet supplemented | 0.14 ± 0.10 (0.11 ± 0.05) | 0.30 (0.17 ± 0.01) | 0.14 ± 0.10 (0.11 ± 0.05) | 0.30 (0.17 ± 0.01) |

**a** The apparent association rate constants were conventionally calculated from the measured k_on and K_d (from Table 1) as k_off = k_on/K_d.

**b** Values were obtained alternatively by stopped-flow quenching of the fluorescence of duplex M/N with AdoMet (Fig. 4, C and D).
a ratio of 1.52. This is a reasonably good agreement, bearing in mind that enzyme-AdoHcy-M/A and enzyme-AdoMet-M/A are analogous but not the same ternary complexes (because we used AdoHcy and non-reactive duplex M/N to avoid the methylation reaction in two different sets of experiments).

The use of duplex M/N with 2-aminopurine in place of the target Ade residue allowed us to carry out a number of accompanying stopped-flow fluorescence experiments, in part to cross-correlate with the results of some of the cross-linking results. For this purpose, we compared the apparent dissociation rate constants determined by single-exponential decay and the apparent rate constants (Table 2). The values determined by the stopped-flow ($k_{on} = 0.17 \mu M^{-1} s^{-1}$ and $k_{off} = 0.11 s^{-1}$) and cross-linking ($k_{on} = 0.3 \mu M^{-1} s^{-1}$ and $k_{off} = 0.14 s^{-1}$) assays were close to each other (Table 2).

**DISCUSSION**

Structure-based Considerations Regarding the Relatively High Yields of Direct Enzyme-Substrate Cross-linking—The photochemical reaction of substrates on dissociation rates, we measured by stopped flow the rate constant of DNA dissociation from the ternary T4Dam-M/N-AdoMet complex. As shown in Fig. 4B, the presence of AdoMet again led to a 150-fold decrease in $k_{off}$ for duplex M/N (0.0051 s$^{-1}$), which is close to the steady-state catalytic turnover constant for the methylation of reactive 2-aminopurine-substituted duplex A/N (0.0036 s$^{-1}$) (13). The next interesting possibility we utilized was the quenching of 2-aminopurine fluorescence in the ternary complex with AdoMet (Fig. 2D), which allowed us to trace the kinetics of AdoMet binding to the preformed enzyme-M/N complex. Experiments were performed at a constant complex concentration and varied cofactor concentrations (0.25–20 μM). The time traces (Fig. 4C) were fitted to a single exponential, and the apparent rate constants obtained were replotted against the concentration of AdoMet (Fig. 4D) to calculate the cofactor association/dissociation rate constants (39). The values determined by the stopped-flow ($k_{on} = 0.17 \mu M^{-1} s^{-1}$ and $k_{off} = 0.11 s^{-1}$) and cross-linking ($k_{on} = 0.3 \mu M^{-1} s^{-1}$ and $k_{off} = 0.14 s^{-1}$) assays were close to each other (Table 2).
Bacteriophage T4 Dam DNA (Adenine-N6)-methyltransferase

Implications for the Mechanism of the T4Dam-catalyzed Reaction—To date, there were no direct experimental data concerning the kinetics of T4Dam-substrate binding. From this study, it follows that the association and dissociation of enzyme-DNA complexes are ~100-fold faster and 10-fold slower, respectively, compared with those for the enzyme-AdoMet (AdoHcy) complexes (Table 2). Nevertheless, bearing in mind that the concentration of cofactor, both in vitro and in vivo (51), is usually 2–3 orders of magnitude higher compared with the concentration of specific DNA, this suggests a random mechanism of formation of the ternary T4Dam-substrate complex. It agrees with the pre-steady-state experimental results indicating random binding of substrates (52); however, the steady-state kinetic data best fit a strictly ordered mechanism (34).

The latter was explained by the assumption that free T4Dam is initially capable of binding randomly to AdoMet and DNA in the first turnover, but after ternary complex formation, the enzyme adopts a conformation with altered substrate binding capabilities and specifically adapted to catalysis. During subsequent turnovers, T4Dam acts according to a strictly ordered reaction mechanism of substrate binding and product release according to the sequence AdoMet ↓ DNA ↓ DNA^Me ↑ AdoHcy ↑. In the case of the homologous E. coli Dam MTase, opposite preferential orders of substrate binding were reported (15, 53). Interestingly, the rates of AdoMet association/dissociation with the isomerized form of T4Dam (k_{on} = 0.13 μM^{-1} s^{-1} and k_{off} = 0.15 s^{-1}) determined by steady-state kinetic analysis (34) are close to those measured in this study for the ternary complex (Table 2). However, the above order of product release contradicts the present data and apparently needs reconciling. In fact, the rates of DNA dissociation are 10-fold slower compared with those for AdoMet (AdoHcy), and the rate of DNA dissociation from the ternary complex is almost equal to the catalytic turnover constant (0.013 s^{-1} versus 0.015 s^{-1}; see “Results”). This suggests another preferential order of product release, viz. AdoHcy ↑ DNA^Me ↑, where DNA^Me ↑ is the limiting step of reaction turnover.

Furthermore, we have shown that both specific DNA and AdoMet (or AdoHcy) reciprocally improve substrate affinity for the enzyme in the ternary complex, significantly reducing rates of dissociation (up to 2 orders of magnitude) (Table 2). From this, we draw two interesting conclusions. First, chemical compounds specifically blocking enzyme binding with one of the reaction substrates will possess a “double” action in lowering the affinity of the enzyme for the second substrate. This is important, bearing in mind the actual problem of regulation of Dam MTase activity in vivo. Second, the dissociation of a long-living ternary complex (disregarding the order of substrates/products release) should be an almost cooperative process, i.e., after dissociation of one of ligands from the enzyme, the second ligand quickly dissociates, too.
The next point concerns the previously proposed mechanism of AdoMet-induced reorientation of T4Dam to the productive strand in a hemimethylated palindromic target site, which was strongly supported by previous 2-aminopurine fluorescence studies and extensive pre-steady-state kinetic analysis (see Refs. 13, 14, and 54 for a detailed discussion). In particular, we revealed that the binding of AdoMet (not AdoHcy or sinefungin) to a preformed complex of T4Dam with DNA duplexes M/N and A/N having asymmetrical substitution of one of the target Ade residues with 2-aminopurine (N) leads to significant quenching of the fluorescence of the latter. On the other hand, the fluorescence of symmetrically modified duplex N/N is not affected by AdoMet addition. This distinction is reasonably explained by the proposed AdoMet-induced reorientation of T4Dam (13). However, such a change in orientation between the DNA strands could be achieved either by repetitive rapid dissociation/re-association or by reorientation without dissociation of T4Dam from the specific DNA. The present stopped-flow analysis of AdoMet binding to the binary T4Dam:M/N complex (Fig. 4, C and D) allowed us to estimate the lower limit for the rate of enzyme reorientation. It should be $\geq 3 \text{s}^{-1}$, i.e. near or faster than the rate constant of fluorescence quenching at the highest AdoMet concentration (20 $\mu$M) that we used in these experiments. From this, we conclude that enzyme reorientation may well occur without physical dissociation of the T4Dam-DNA complex because its dissociation rate constant is significantly lower (0.5 $\text{s}^{-1}$ in the absence and 0.013 $\text{s}^{-1}$ in the presence of a methyl donor analog) (Table 2). In fact, the structural data available now demonstrate three prominent orientations of T4Dam MTase relative to the DNA axis along the site recognition pathway; they suggest that the enzyme molecule is able to rotate as a rigid body relative to the DNA axis (9).

**Prospects for Direct Rapid Laser UV Cross-linking**—Using T4Dam as a model MTase, we found some general advantages of the rapid laser UV cross-linking technique. First, it introduces “zero length” covalent bonds and does not require pre-incorporation of either photoreactive or chemically reactive modifiers or fluorescent reporters, which potentially may distort the structural/mechanistic characteristics of natural substrates. Second, it permits enzyme binding studies with both specific DNA and AdoMet. One of the interesting possibilities arising herein is to measure the equilibrium binding constants for natural substrates in the pre-reactive ternary complex because rapid laser UV cross-linking allows “freezing” of such a complex at appropriate time intervals following binding but prior to the subsequent chemical step. Third, it allows investigation of the parameters of enzyme-substrate binding not only at equilibrium, but, to a certain extent, in real time. Thus, direct rapid laser UV cross-linking is well suited for MTase binding studies under non-perturbing native conditions and combines several favorable characteristics in one universal approach. To test the potential of this approach, in this study, we measured a number of parameters of T4Dam-substrate binding, some of which were inaccessible directly by other means (such as dissociation rates for the binary T4Dam:AdoMet complex and for enzyme complexes with non-fluorescent canonical duplex M/A). Nevertheless, the technique does have some inherent disadvantages. First is the complexity of the method in obtaining detailed transient binding data compared with stopped-flow approaches producing fast and accurate time traces consisting up to 1000 data points; however, the stopped-flow technique requires incorporation of fluorophore into DNA and is not always applicable because of the absence or insufficient amplitude of fluorescence signal changes. Second is the necessity of the use of environmentally dangerous radiolabeled ligands. Finally, the technique is limited by the low quantum yield of cross-linking. In this respect, it should be noted that some of the T4Dam amino acids interacting and possibly cross-linking with DNA (Fig. 5A) are residues conserved among numerous $\alpha$-group (adenine-$N^6$)-MTases (47). Moreover, bound AdoMet is often surrounded by a number of aromatic residues; prolonged irradiation, even at low energy UV light, fixes MTase-AdoMet complexes with good cross-linking yields (19–25). In conclusion, the results from this study suggest that the rapid laser UV cross-linking technique is a useful addition to the arsenal of tools used to study MTases and may even be the method of choice to probe MTase-substrate interactions in those cases in which the desired data are not accessible by other means.

Acknowledgments—The help and support of Emeline Bouffartigue and Sylvie Rimsky are greatly appreciated. We thank Profs. Olga S. Fedorova and Vladimir V. Koval for expert assistance and access to a stopped-flow facility.

**REFERENCES**

1. Jeltsch, A. (2002) ChemBioChem 3, 274–293
2. Lemenaika, S., Reddy, Y. V., and Rao, D. N. (2006) Biochem. J. 399, 177–190
3. Barras, F., and Marinus, M. G. (1989) Trends Genet. 5, 139–143
4. Casadesus, J., and Low, D. (2006) Microbiol. Mol. Biol. Rev. 70, 830–856
5. Mashboon, N., Pruss, C., Carroll, M., Johnson, P. H., and Reich, N. O. (2006) J. Biomol. Screen. 11, 497–510
6. Schlagman, S. L., and Hattman, S. (1983) Gene (Amst.) 22, 139–156
7. Hattman, S., and Malygin, E. G. (2004) Prog. Nucleic Acid Res. Mol. Biol. 77, 67–126
8. Yang, Z., Horton, J. R., Zhou, L., Zhang, X. J., Dong, A., Zhang, X., Schlagman, S. L., Kossykh, V., Hattman, S., and Cheng, X. (2003) Nat. Struct. Biol. 10, 849–855
9. Horton, J. R., Liebert, K., Hattman, S., Jeltsch, A., and Cheng, X. (2005) Cell 121, 349–361
10. Malygin, E. G., Petrov, N. A., Gorbunov, Y. A., Kossykh, V. G., and Hattman, S. (1997) Nucleic Acids Res. 25, 4393–4399
11. Zinoviev, V. V., Evdokimov, A. A., Gorbunov, Y. A., Malygin, E. G., Kossykh, V. G., and Hattman, S. (1998) Biol. Chem. 379, 481–488
12. Malygin, E. G., Zinoviev, V. V., Petrov, N. A., Evdokimov, A. A., Jen-Jacobsen, L., Kossykh, V. G., and Hattman, S. (1999) Nucleic Acids Res. 27, 1135–1144
13. Malygin, E. G., Evdokimov, A. A., Zinoviev, V. V., Ovechkina, L. G., Lindstrom, W. M., Jr., Reich, N. O., Schlagman, S. L., and Hattman, S. (2001) Nucleic Acids Res. 29, 2361–2369
14. Malygin, E. G., Sclavi, B., Zinoviev, V. V., Evdokimov, A. A., Hattman, S., and Buckle, M. (2004) J. Biol. Chem. 279, 50012–50018
15. Urig, S., Gowher, H., Hermann, A., Beck, C., Fatemi, M., Humeny, A., and Jeltsch, A. (2002) J. Mol. Biol. 319, 1085–1096
16. Hockensmith, J. W., Kubasek, W. L., Vorachek, W. R., and von Hippel, P. H. (1986) J. Biol. Chem. 261, 3512–3518
17. Buckle, M., Geiselmam, J., Kolb, A., and Buc, H. (1991) Nucleic Acids Res. 19, 833–840
18. Dimitrov, S. I., and Moss, T. (2001) Methods Mol. Biol. 148, 395–402
Bacteriophage T4 Dam DNA (Adenine-N6)-methyltransferase

19. Som, S., and Friedman, S. (1990) J. Biol. Chem. 265, 4278–4283
20. Reich, N. O., and Everett, E. A. (1990) J. Biol. Chem. 265, 8929–8934
21. Wenzel, C., and Guschlbauer, W. (1993) Nucleic Acids Res. 21, 4604–4609
22. Powell, L. M., Dryden, D. T., Willcock, D. F., Pain, R. H., and Murray, N. E. (1993) J. Mol. Biol. 234, 60–71
23. Ahmad, I., and Rao, D. N. (1994) Gene (Amst.) 142, 67–71
24. Finta, C., Sulima, U., Venetianer, P., and Kiss, A. (1995) Gene (Amst.) 164, 65–69
25. Adams, G. M., and Blumenthal, R. M. (1997) Biochemistry 36, 8284–8292
26. Kossykh, V. G., Schlagman, S. L., and Hattman, S. (1995) J. Biol. Chem. 270, 14389–14393
27. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
28. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
29. Copeland, R. A. (2000) Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, Wiley-VCH, New York
30. Lindstrom, W. M., Jr., Flynn, J., and Reich, N. O. (2000) J. Biol. Chem. 275, 4912–4919
31. Vilkaitis, G., Merkienė, E., Serva, S., Weinhold, E., and Klimasauskas, S. (2001) J. Biol. Chem. 276, 20924–20934
32. Szegedi, S. S., Reich, N. O., and Gumpert, R. I. (2000) Nucleic Acids Res. 28, 3962–3971
33. Szczelkun, M. D., and Connolly, B. A. (1995) Biochemistry 34, 10724–10733
34. Evdokimov, A. A., Zinoviev, V. V., Malygin, E. G., Schlagman, S. L., and Hattman, S. (2002) J. Biol. Chem. 277, 279–286
35. Everett, E. A., Falick, A. M., and Reich, N. O. (1990) J. Biol. Chem. 265, 17713–17719
36. Maegley, K. A., Gonzalez, L. J., Smith, D. W., and Reich, N. O. (1992) J. Biol. Chem. 267, 18527–18532
37. Schlucekber, G., Kozak, M., Bleimling, N., Weinhold, E., and Saenger, W. (1997) J. Mol. Biol. 265, 56–67
38. Tuzikov, F. V., Tuzikova, N. A., Naumochkin, A. N., Zinoviev, V. V., and Malygin, E. G. (1997) Mol. Biol. (Engl. Transl. Mol. Biol. (Mosc)) 31, 73–76
39. Fersht, A. (1985) Enzyme Structure and Mechanism, W. H. Freeman & Co., New York
40. Vilkaitis, G., Dong, A., Weinhold, E., Cheng, X., and Klimasauskas, S. (2000) J. Biol. Chem. 275, 38722–38730
41. Liebert, K., Hermann, A., Schlickenrieder, M., and Jeltsch, A. (2004) J. Mol. Biol. 341, 443–454
42. Allan, B. W., Reich, N. O., and Beechem, J. M. (1999) Biochemistry 38, 5308–5314
43. Beck, C., and Jeltsch, A. (2002) Biochemistry 41, 14103–14110
44. Pemberton, I. K., Buckle, M., and Buc, H. (1996) J. Biol. Chem. 271, 1498–1506
45. Chodosh, L. A. (1988) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp 12.2.1–12.2.10 John Wiley & Sons, Inc., New York
46. Horton, J. R., Liebert, K., Bekes, M., Jeltsch, A., and Cheng, X. (2006) J. Mol. Biol. 358, 559–570
47. Malone, T., Blumenthal, R. M., and Cheng, X. (1995) J. Mol. Biol. 253, 618–632
48. Holz, B., Dank, N., Eickhoff, J. E., Lipps, G., Krauss, G., and Weinhold, E. (1999) J. Biol. Chem. 274, 15066–15072
49. Wong, D. L., and Reich, N. O. (2001) Biochemistry 39, 15410–15417
50. Pingoud, A., Geyer, H., Geyer, R., Kubareva, E., Bujnicki, J. M., and Pingoud, A. (2005) Mol. Biosyst. 1, 135–141
51. Posnick, L. M., and Samson, L. D. (1999) J. Bacteriol. 181, 6756–6762
52. Malygin, E. G., Lindstrom, W. M., Jr., Schlagman, S. L., Hattman, S., and Reich, N. O. (2000) Nucleic Acids Res. 28, 4207–4211
53. Mashhoon, N., Carroll, M., Pruss, C., Eberhard, J., Ishikawa, S., Estabrook, R. A., and Reich, N. (2004) J. Biol. Chem. 279, 52075–52081
54. Zinoviev, V. V., Evdokimov, A. A., Malygin, E. G., Schlagman, S. L., and Hattman, S. (2003) J. Biol. Chem. 278, 7829–7833