Escherichia coli DNA Adenine Methyltransferase

THE STRUCTURAL BASIS OF PROCESSIVE CATALYSIS AND INDIRECT READ-OUT

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We have investigated the structural basis of processive GATC methylation by the Escherichia coli DNA adenine methyltransferase, which is critical in chromosome replication and mismatch repair. We determined the contribution of the orthogradically conserved phosphate interactions involving residues Arg95, Asn126, Asn132, Arg116, and Lys139, which directly contact the DNA outside the cognate recognition site (GATC) to processive catalysis, and that of residue Arg137, which is not conserved and contacts the DNA backbone within the GATC sequence. Alanine substitutions at the conserved positions have large impacts on processivity yet do not impact $k_{\text{cat}}/K_{\text{DNA}}$ or DNA affinity ($K_{\text{DNA}}$). However, these mutants cause large preferences for GATC sites varying in flanking sequences when considering the pre-steady state efficiency constant $k_{\text{chem}}/K_{\text{DNA}}$. These changes occur mainly at the level of the methylation rate constant, which results in the observed decreases in processive catalysis. Thus, processivity and catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) are uncoupled in these mutants. These results reveal that the binding energy involved in DNA recognition contributes to the assembly of the active site rather than tight binding. Furthermore, the conserved residues (Arg95, Asn126, Asn132, and Arg116) repress the modulation of the response of the enzyme to flanking sequence effects. Processivity impacted mutants do not show substrate-induced dimerization as is observed for the wild type enzyme. This study describes the structural means by which an enzyme that does not completely enclose its substrate has evolved to achieve processive catalysis, and how interactions with DNA flanking the recognition site alter this processivity.

Processive enzyme catalysis, whereby a single substrate binding event is coupled to multiple rounds of catalytic turnovers, occurs frequently with enzymes that act on polymeric substrates such as DNA and in many cases, is essential for the viability of the organism (1–5). A recent review parses processive enzymes into two structural groups: those that completely enclose their substrate, or those that partially enclose their substrate by forming a “saddle” or hand shape around it (1). Although the structural means by which an enzyme that completely encloses its substrate achieves processive catalysis is intuitively understood, the structural basis for processive catalysis for enzymes that only partially enclose their substrates is less obvious, particularly because structurally similar enzymes show diverse levels of processivity (1). For example, although the extensive protein-DNA interface observed in this latter grouping may contribute to processive kinetics, many non-processive (distributive) enzymes such as some restriction endonucleases share the saddle or hand-shaped interface (6–8).

The involvement of Escherichia coli DNA adenine methyltransferase (EcoDam) in diverse biological pathways provides a compelling system in which to study the mechanism and biological significance of processivity. EcoDam methylates the N-6 position of adenine in the DNA sequence 5′-GATC-3′ and is highly processive, catalyzing multiple methyltransfer priors prior to dissociating from the DNA (9, 10). Unlike most bacterial methyltransferases, EcoDam does not belong to a restriction-modification system that serves to protect the cell from foreign DNA. Instead, the methylation by EcoDam of the ~20,000 GATC sites within the E. coli genome is critical in a number of biological pathways including gene regulation, chromosome replication, mismatch repair, and nucleoid structure determination (11, 12). It has been shown that there are relatively few EcoDam molecules per bacterial cell suggesting that high processivity is essential in the methylation of the large number of GATC sites in the E. coli genome to avoid hyper-mutating phenotypes (10, 11, 13). However, EcoDam involvement in some cellular pathways lacks any need for processivity, and in some circumstances the enzyme is optimized for the lack of such activity (14). Gene expression and proteomic studies of bacteria in which the EcoDam gene has been deleted show dramatic and widespread changes in RNA and protein levels, in many cases involving well characterized virulence factors (15–18). Furthermore, a growing number of bacterial pathogens that contain an EcoDam analog require adenine methylation for virulence (19). The inhibition of EcoDam has been proposed and pursued as a viable antibiotic strategy (20, 21) because humans lack this activity.

Exocyclic adenine methyltransferases can be separated into six classes ($\alpha$, $\beta$, $\gamma$, $\zeta$, $\delta$, and $\epsilon$) based on the arrangements of the AdoMet binding domains (X, I, II, and III) and catalytic domains (III-VIII) in relation to the target recognition domain (TRD) (22, 23). EcoDam belongs to the $\alpha$-class of exocyclic methyltransferases, as do orthologs such as M.EcoRV and T4 Dam (22). The crystal structure of EcoDam (24) shows two distinct domains: a DNA binding domain consisting of a five-helix bundle and a $\beta$-hairpin loop, and a seven-stranded cata-

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2 The abbreviations used are: EcoDam, Escherichia coli DNA adenine methyltransferase; AdoMet, S-adenosylmethionine; TRD, target recognition domain; WT, wild type; BSA, bovine serum albumin.
EcoDam. This work is directly relevant to our recent demonstration that the wild type enzyme shows complex kinetic behavior involving substrate-dependent activation and dimerization, both of which are coupled to processivity. This work offers insight into the structural basis of processive catalysis for an enzyme that does not completely enclose the DNA in addition to enhancing our understanding of indirect read-out by DNA modifying enzymes.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis, Enzyme Expression, and Purification—**

Six EcoDam mutants were produced (Arg137 → Ala, Arg116 → Ala, Arg95 → Ala, Asn126 → Ala, Asn132 → Ala, and Lys139 → Ala) using the QuickChange PCR mutagenesis kit (Stratagene) with vector pDal572 as a template and six sets of primers (Operon). The resulting PCR products were digested with DpnI to remove the WT plasmid and transformed into XL2 Blue (Stratagene) E. coli cells. WT EcoDam and mutants were expressed and purified as previously described (25). In brief, cells containing the desired construct were grown at 37 °C in LB media supplemented with 25 μg/ml kanamycin and 12.5 μg/ml tetracycline. Once an A600 of 0.4–0.6 was reached, cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and 0.05% l-arabinose and grown for 2 h at 37 °C. Pelleted cells were resuspended in 40–60 ml of P-11 buffer (50 mM potassium phosphate buffer, pH 7.4, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 M NaCl, 10% glycerol) and lysed by French press. The lysate was centrifuged at 15,000 × g for 60 min at 4 °C and the supernatant loaded onto a 60-ml phosphocellulose (Whatman) column. The protein was eluted with a salt gradient between 0.2 and 0.8 M NaCl and those fractions containing EcoDam were pooled and dialyzed in BS buffer (20 mM potassium phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol). The dialyzed protein was loaded onto a 20-ml Blue Sepharose 6 Fast Flow (GE Healthcare) column pre-equilibrated in BS buffer and protein

lytic domain (Fig. 1A). The structure reveals an incomplete enclosure of the DNA in which most of the base-specific contacts occur through the major groove, whereas the DNA is positioned via direct and water-mediated phosphate interactions (24). This lack of structural enclosure and large DNA interface places EcoDam in the second structural group of processive enzymes (1) thus making the basis for its highly processive nature poorly understood.

We previously showed that EcoDam processivity and differential methylation of biologically derived GATC sites is in part regulated by the DNA immediately flanking the target site (14, 25). This “higher order” specificity modulates the ability of the enzyme to methylate a particular sequence and act processively, and is important to the function of the enzyme in several biological pathways. Here we describe the structural basis for both processive catalysis and preferential site methylation for EcoDam.
The Structural Basis of Processivity

eluted with a salt gradient between 0 and 1.5 M NaCl. Fractions containing the WT and mutant enzymes were flash frozen and stored at −80 °C. Concentrations were determined using the extinction coefficient 1.16 mg cm−1 at 280 nm.

DNA Substrates—All DNA oligonucleotides were ordered from Operon and resuspended in TE (10 mM Tris, pH 7.5, 1 mM EDTA). All constructs were annealed in a 1:1 molar ratio by heating at 95 °C for 10 min followed by slow cooling (~5 h) to 22 °C. Proper product formation was verified by PAGE. The preferred substrate (P) consisted of the annealed product of the oligonucleotides 5’-CATTTAAGAGCTGAATGCTGTTTGCCGGATCAAGTAAATG-3’ and 5’-GGCATAGGCGTAGA-GCATACCGGATCAAGTAAATG-3’, whereas the non-preferred substrate (N-P) consisted of the annealed product of the oligonucleotides 5’-CATTAGACGATCTTTATGC-3’ and 5’-GCATATAAGATCGTCTAAATG-3’, respectively. Two 58-bp oligonucleotides with the sequences 5’-H11032 and 5’-H11032 were annealed to form the substrate for processivity analysis. Concentrations of all DNA constructs were determined by measuring the A260.

KD,DNA Determination—Anisotropy experiments were performed on a Fluoromax-2 fluorimeter (ISA SPEX) equipped with an L-format autopolarizer at 22 °C. The change in anisotropy was measured (fluorescein) duplex DNA in MRB (100 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mg/ml BSA) supplemented with 50 μM sinefungin was measured by a filter binding assay as previously described (33). EcoDam and mutants were diluted into protein dilution buffer (20 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.2 mg/ml BSA, 2 mM dithiothreitol, 10% glycerol). Reactions were carried out at 22 °C with limiting enzyme in MRB supplemented with 0.2 mg/ml BSA. WT and mutant enzymes were diluted into protein dilution buffer (20 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mg/ml BSA, 2 mM dithiothreitol, 10% glycerol). Reactions were carried out at 22 °C and contained 2–5 nM enzyme in MRB, 0.2 mg/ml BSA, 25 μM tritiated AdoMet (PerkinElmer Life Sciences), and DNA (0–500 nM) in a final volume of 20 μl. Mixtures were equilibrated at 22 °C prior to initiation with DNA. Reactions were quenched with 10 μl of 1% SDS at a single time point (30–60 min dependent upon activity) and 25 μl was spotted onto 2.5-cm Whatman DE81 circular filter papers. Filter papers were washed three times in 50 mM KH2PO4, once in 80% ethanol, once in 100% ethanol, and once in diethyl ether for 5 min each. Papers were dried and submerged in Biosafell scintillation fluid. Tritium levels were quantitated in a Beckman Coulter LS6500 scintillation counter. Counts were converted to methylated product per unit time and plotted against the DNA concentration. Values for kapp,DNA and kcat were found by fitting the data to a rectangular hyperbola in Sigma Plot 6.1.

Burst Analysis—Reactions were carried out in MRB with 50 nM enzyme, 500 nM DNA, and 30 μM tritiated AdoMet at 22 °C. Reactions were quenched by placing 10-μl aliquots of the reaction into an equal volume of 1% SDS at time points 0, 15, 30, 45, 60, 90, and 120 s or 0, 1, 5, 10, 30, 60, and 120 min depending upon reactivity of the enzyme. 15 μl of the resulting mixture was spotted on DE81 filter papers and washed as described above. Tritium levels were quantitated, converted to methylated product, and plotted against time in Sigma Plot. The kapp,DNA (not shown) was found by fitting the data to a linear curve and dividing the slope of the line by the enzyme concentration. This value was comparable with the actual kcat of WT and mutant enzymes. The y intercept of the linear fit to the data represents the burst magnitude.

Single Turnover Kinetics—for the WT enzyme and R137A mutant, single turnover measurements to determine kchem were performed by using a rapid chemical-quench flow apparatus (RQF-3, Kin Tek Instruments, University Park, PA) with saturating enzyme and AdoMet (420 nM and 30 μM) and limiting DNA (288 nM) in MRB. The temperature was maintained at 22 °C for all experiments. Enzyme and substrates in two 20-μl sample loops were mixed rapidly into a single reaction loop of specified dimensions to control the time of the reaction. Reactions were quenched with 1% SDS at 0, 0.2, 0.5, 1, 2, 3, 5, 10, and 30 s. At least three data points from each time point were measured and averaged. All other single turnover assays were completed at 22 °C with limiting DNA and excess EcoDam and AdoMet. Reactions took place in MRB with 0.2 mg/ml BSA, 288 nM DNA, 420 nM EcoDam, and 30 μM AdoMet in a total volume of 100 μl. All reactions were initiated with addition of DNA. At 0, 15, 30, 60, 90, 120, 180, 300, and 600 s 10-μl aliquots of the reaction were removed and quenched in 10 μl of 1% SDS. 15 μl of the resulting mixture was placed on DE81 filter papers. Samples were washed, dried, and counted as described above. Counts were converted to nanomolar methylated product and plotted against time. The kchem for each substrate was found by fitting the data to a single exponential in Sigma Plot 6.1.

Processivity Assay—Processivity assays were carried out at 22 °C with limiting enzyme in MRB supplemented with 0.2 mg/ml BSA. WT and mutant enzymes were diluted into protein dilution buffer so that each reaction contained 10 nM enzyme, 600 nM DNA, and 30 μM AdoMet. Reactions were quenched by removing aliquots from the reaction mixture into pre-heated TE at 75 °C, and incubated for at least 15 min to ensure heat inactivation of EcoDam. After cooling to room temperature, samples were digested with DpnII for at least 12 h at 37 °C. A 10-μl ladder (Invitrogen), an uncut control, and the digested samples were run on 12 (58-bp substrate) or 8% (270-bp substrate) native polyacrylamide gels for 4 h at 250 V. Gels were stained with SYBR Au (Invitrogen) and scanned on a Storm 840 PhosphorImager (Amersham Biosciences). Band density was determined using ImageQuant version 1.2 (GE Healthcare) and further analyzed in Microsoft Excel. Density changes with time were finally plotted in Sigma Plot 6.1. The slope of the linear fit...
The Structural Basis of Processivity

FIGURE 2. Structure based sequence alignment of a portion of the target recognition domain of EcoDam (PDB 2G1P) and selected orthologs. Sequences used for the alignment and their EMB accession numbers are as follows: EcoDam (AAG58487.1), M.MboIA (BAA03071.1), M.MjaIII (AAB98590.1), M.Cvi BI (AAAB88829.1), M.EcoRV (CAA25209.1), and M.DpnIIA (AAA268721), K. pneumoniae (ABR79145.1), S. enterica (CAD08130.1), M.SmalI (CAA55177.1), Y. pestis (CAL18840.1), V. cholerae (AAF95767.1), T4Dam (AAD42553.1), M.HindIV (AAC21877.1), and N. meningitides (AAY52162). Invariant residues (black background, white lettering) and conserved residues (boxed) are shown. Conserved residues directly contacting the phosphate backbone of the DNA flanking the target GATC are indicated with an arrow.

Structural Analysis—PDB file 2G1P containing the EcoDam/DNA co-crystal structure was downloaded from the RCSB data base and analyzed in PyMOL version 0.98 (DeLano Scientific). The target binding domain and the conserved residues within hydrogen bonding distance (≤3.0 Å) of the phosphate groups flanking the target GATC were displayed. Direct hydrogen bonds made from a side chain to the phosphate backbone were found by measuring the distances between atoms. Additional residues within hydrogen bonding distance of conserved residues were displayed and their interactions were measured in the same way as mentioned above. Images were rendered and saved as png files for use in Fig. 9.

RESULTS

A structure-based sequence alignment of a portion of the TRD of EcoDam (PDB code 2G1P) and selected orthologs reveals the conservation of the Arg116, Lys139, and Asn132 residues and the invariant residues Arg95 and Asn126 (Fig. 2). This is consistent with previous structure-based sequence alignments based on the T4 Dam structure (29). Although each of the conserved residues is found within the TRD, none are involved in
direct contacts to the GATC site. Instead, the crystal structure shows that these residues are involved in direct hydrogen bonds to the backbone of DNA surrounding the GATC site (Fig. 1). Arg116 and Lys139 contact two phosphates on the left-hand (5') side of the adenine being methylated, whereas Arg95, Asn126, and Asn132 contact phosphates on the non-target strand of the right-hand (3') side of the target adenine. These residues appear to anchor the -hairpin that contains base-specific interacting residues and this extensive, conserved, protein-DNA interface is thought to aid in the positioning of the enzyme onto DNA so that catalysis can occur (24). Furthermore, the orthologous residues in T4 Dam have been shown to stabilize a nonspecific enzyme-DNA complex (29). This observation, in addition to our previous work, suggests that these residues may aid in the processive methylation of multiple GATC sites in EcoDam in addition to providing an indirect readout mechanism responsible for higher-order specificity (14, 25).

We replaced the residues at the conserved positions with alanines to test their role in processivity, catalysis, and site preference. Additionally Arg137, which contacts the phosphate backbone within the GATC site, was also mutated to alanine to serve as a control. To clarify, although all of the residues targeted for mutagenesis interact with a phosphate on the DNA substrate, they are distinct by virtue of the location of the particular phosphate within or outside of the GATC site. The EcoDam mutants were tested for their ability to processively methylate a 58-bp duplex DNA substrate containing preferred (P) and non-preferred (N-P) GATC sites (Fig. 3A). By monitoring the product formed in the early phases of the reaction (≤25%
The Structural Basis of Processivity

TABLE 1
Steady state efficiency for WT and mutants on a preferred (P) and non-preferred (N-P) substrate

Reactions were performed with limiting enzyme at 22 °C with excess AdoMet and DNA. The steady state efficiency or $k_{\text{cat}}/K_{\text{DNA}}$ (f) for each mutant and substrate is shown. As is seen with the WT enzyme, R137A (control mutant), K139A, R116A, N132A, and R95A show significant perturbations in the $k_{\text{cat}}$ and $K_{\text{DNA}}$ values for each substrate. These changes compensate for each other and the overall $f/P_{\text{chem}}$ remains close to 1. Thus, any site preference or change in methylation efficiency due to the lack of a conserved phosphate interaction is not revealing itself under these conditions. However, the N126A did not detectably methylate the non-preferred substrate while maintaining WT-like steady state parameters on the preferred substrate. This result suggests that the invariant Asn$^{61}$ residue is aiding decreasing the WT responsiveness to the DNA surrounding the GATC site.

| N-P, 5'-GCATAAAAGATCGTCATAAATG-3' | \(K_{\text{DNA}}\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_{\text{DNA}}\) |
|------------------------------------|----------------|---------------|-----------------|
| WT | 81 ± 4 | 0.91 ± 0.03 | 1.12 ± 0.07 |
| R137A | 220 ± 30 | 3.5 ± 0.4 | 1.57 ± 0.05 |
| K139A | 27 ± 5 | 0.49 ± 0.06 | 1.8 ± 0.1 |
| R116A | 7 ± 4 | 0.054 ± 0.002 | 0.8 ± 0.3 |
| N132A | 10 ± 2 | 0.10 ± 0.01 | 0.96 ± 0.05 |
| R95A | 8 ± 5 | 0.049 ± 0.007 | 0.7 ± 0.3 |
| N126A | ND | ND | ND |

| P, 5'-GCATACCCGGATCAAGTAAAATG-3' | \(K_{\text{DNA}}\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_{\text{DNA}}\) |
|----------------------------------|----------------|---------------|-----------------|
| WT | 84 ± 13 | 4 ± 10 | 5 ± 1 |
| R137A | 47 ± 8 | 7 ± 1 | 14 ± 4 |
| K139A | 150 ± 40 | 3.9 ± 0.9 | 3 ± 1 |
| R116A | 71 ± 6 | 0.020 ± 0.001 | 0.028 ± 0.003 |
| N132A | 210 ± 40 | 0.027 ± 0.005 | 0.013 ± 0.004 |
| R95A | 60 ± 1 | ±0.005 ± 0.001 | 0.008 ± 0.002 |
| N126A | 84 ± 16 | ±0.002 ± 0.001 | 0.003 ± 0.001 |

| N-P, 5'-GCATAAAAGATCGTCATAAATG-3' | \(f/P_{\text{chem}}\) | \(f/P_{\text{chem}}\) |
|------------------------------------|----------------|-----------------
| WT | 9 ± 2 | 8.6 ± 0.2 |
| R137A | 9 ± 2 | 8.6 ± 0.2 |
| K139A | 14 ± 4 | 37 ± 13 |
| R116A | 13 ± 4 | 2.6 ± 0.8 |
| N132A | 13 ± 4 | 2.6 ± 0.8 |
| R95A | 10 ± 1 | 0.7 ± 0.1 |
| N126A | 10 ± 1 | 0.7 ± 0.1 |

| P, 5'-GCATACCCGGATCAAGTAAAATG-3' | \(f/P_{\text{chem}}\) | \(f/P_{\text{chem}}\) |
|----------------------------------|----------------|-----------------
| WT | 9 ± 2 | 8.6 ± 0.2 |
| R137A | 9 ± 2 | 8.6 ± 0.2 |
| K139A | 14 ± 4 | 37 ± 13 |
| R116A | 13 ± 4 | 2.6 ± 0.8 |
| N132A | 13 ± 4 | 2.6 ± 0.8 |
| R95A | 10 ± 1 | 0.7 ± 0.1 |
| N126A | 10 ± 1 | 0.7 ± 0.1 |

$^a$ ND, not determined.

TABLE 2
Pre-steady state efficiency constants for the preferred (P) and non-preferred substrates (N-P)

$K_{D_{\text{even}}}$ values for WT and mutants were obtained by fluorescence anisotropy when protein is titrated into a constant amount of DNA with saturating sinefungin in reaction buffer. Single turnover reactions with limiting DNA and excess enzyme and AdoMet were fit to a simple exponential to obtain $K_{D_{\text{even}}}$ for WT and mutant enzymes on each substrate. When compared to WT on the non-preferred (N-P) substrate, the N126A and R95A showed the largest decrease in overall efficiency $\langle f/P_{\text{chem}}\rangle$ for each substrate as the processivity (completion) we can describe the fraction of enzymatic encounters that result in double methylation events as the processivity factor ($f_P$) and the site preference on this substrate ($E_P/E_{N-P}$) as previously described (25–27). For example, Stanford et al. (27) demonstrated that the endonuclease EcoRV endonuclease is less likely to processively cleave adjacent cognate sites as the distance between them increases. They observed that when EcoRV sites were placed 54 bp apart, the $f_P$ value is about 0.4; whereas when the distance between the sites increased to 764 bp, the $f_P$ value decreased to 0.1. In general, a highly processive enzyme would have a predicted $f_P$ value around 1, whereas a distributive enzyme would have an $f_P$ value close to zero. As shown in Fig. 3B, WT EcoDam methylates the 58-bp duplex in a high processively with an $f_P$ of ~1 (Fig. 3D). Only a small amount of intermediates corresponding to single methylation events at the preferred or non-preferred sites could be detected above background levels. Therefore, little to no site preference is revealed on this substrate. In comparison with the WT enzyme, all mutants showed a decrease in processivity on the 58-bp substrate; however, K139A and control R137A showed the smallest variability from WT with $f_P$ values equal to 0.73 and 0.58, respectively. This small change in processivity still results in a majority of binding events producing methylation at both sites and is minimal when compared with the conserved flanking interacting mutants whose processivity is decreased substantially (Fig. 3D). Under these conditions, the R116A and N126A mutants showed the greatest decrease in processive methylation with $f_P$ values of 0.10 and 0.13, respectively, in addition to the largest discrimination between the P and N-P substrates with $E_P/E_{N-P}$ values of 18.75 and 16.50, respectively (Fig. 3).

To identify the kinetic step responsible for the poor processivity of R116A, N126A, R95A, and N132A, we kinetically characterized all mutants using two DNA substrates based on the preferential or non-preferential methylation by EcoDam (25). An A-tract positioned 5' to the GATC (N-P) was shown to decrease methylation kinetics and exacerbate preferential methylation for WT EcoDam when compared with a GATC without an A-tract (P). These substrates were used to determine the steady state activity of each mutant (Table 1) as well as the pre-steady state kinetics (Table 2). Under steady state conditions, the concentrations of the mutant enzymes are limiting and the DNA and AdoMet are in excess. The $k_{\text{cat}}$ and $k_{\text{DNA}}$ for the mutants with the N-P substrate vary 19- and 12-fold, respectively, when compared with the WT enzyme (Table 1). The exception is N126A, which had undetectable levels of activity with the N-P substrate. The overall efficiency constant ($k_{\text{cat}}/K_{\text{DNA}}/E_{\text{chem}}$) for N-P remains relatively unchanged from the WT EcoDam, due to the compensatory changes in each parameter. For example, N132A shows an approximate 8-fold decrease in both $K_{\text{DNA}}$ and $k_{\text{cat}}$ for the N-P substrate, leaving $k_{\text{cat}}/K_{\text{DNA}}$ unchanged.
The Structural Basis of Processivity

FIGURE 4. Flanking mutants are dramatically affected in \( k_{\text{chem}} \). A, graphical analysis of steady state and pre-steady state kinetic data on N-P from Tables 1 and 2. The log of the fold-change (without regard to whether this change was positive or negative) from WT for each mutant studied was plotted against each kinetic parameter. It is easily observed that whereas perturbations in steady state parameters exist; changes in \( k_{\text{cat}} \) and \( K_m \) compensate for each other thus equilibrating the overall efficiency constant (\( k_{\text{cat}}/K_m \)). However, this does not occur under pre-steady state conditions where \( k_{\text{chem}} \) is dramatically affected resulting in large changes in the pre-steady state efficiency constant (\( k_{\text{chem}}/K_m \)). B, graphical analysis of steady state and pre-steady state kinetic data on P from Tables 1 and 2. Again, the log of the fold-change from WT on the P substrate for each mutant was plotted against each kinetic parameter. Compensatory changes in steady state kinetic constants are also apparent with P resulting in only moderate changes in the overall \( k_{\text{cat}}/K_m \) for each mutant. Although to a lesser extent, \( k_{\text{chem}} \), is also the most dramatically affected kinetic parameter for the P substrate resulting in large deviations from the WT \( k_{\text{chem}}/K_m \).

from that of the WT enzyme (Table 1, Fig. 4). Mutants show similar behavior on P with 5- and 4-fold variations in \( k_{\text{cat}} \) and \( K_m \)DNA in relation to the WT enzyme. In this case, N126A is no exception; any change in \( k_{\text{cat}} \) is compensated for by a change in \( K_m \)DNA thus equilibrating the overall \( k_{\text{cat}}/K_m \)DNA or \( k_{\text{chem}} \) for the mutants and WT enzyme (Table 1, Fig. 4).

We previously demonstrated that WT EcoDam activity is highly responsive to the DNA surrounding its cognate site using a combination of kinetic approaches (25). However, preference for one GATC site over another did not reveal itself in the steady state efficiency constant; instead, the preference was visible in the \( k_{\text{chem}} \) catalytic step. To see if this characteristic is shared by the mutants, we first compared the steady state efficiency constants for P (\( \xi_p \)) and N-P (\( \xi_{\text{N-P}} \)) substrates. The ratio of the two constants \( \xi_p/\xi_{\text{N-P}} \) allows for a direct comparison of the efficiency of the enzyme at either site; thus, we observe \( \xi_p/\xi_{\text{N-P}} = 1 \) for the WT enzyme showing no preference for GATC sites varying in flanking sequences (Table 1). This results from compensatory changes in both \( k_{\text{cat}} \) and \( K_m \)DNA. For example, the \( k_{\text{cat}} \) for N-P is 4-fold faster than that of P and the \( K_m \)DNA for N-P is 4-fold higher than that of P (Table 1, Fig. 4).

Similar compensatory trends that equilibrate the \( \xi_p/\xi_{\text{N-P}} \) to \( \sim 1 \) are apparent with the mutant enzymes, with the exception of the N126A mutant. Due to the inability of N126A to detectably methylate the N-P substrate, the \( \xi_p/\xi_{\text{N-P}} \) could not be determined (Table 1). The data for N126A on the P substrate reveals kinetic parameters unlike the other mutants; its \( K_m \)DNA is essentially equal to that of the WT enzyme, yet \( k_{\text{cat}} \) is decreased \( \sim 3 \)-fold. For all other mutants, the \( K_m \)DNA for the P substrate is slightly increased, whereas the \( k_{\text{cat}} \) is also increased. Based on this variant behavior and the inability to detect methylation on the N-P substrate, we suggest that the \( \xi_p/\xi_{\text{N-P}} \) for this mutant would be higher than the maximum of 3 that we observed. Therefore, N126A is the most responsive to the DNA flanking a GATC site implicating this residue is of particular importance when considering indirect read-out.

Using pre-steady state burst kinetic analysis we qualitatively investigated the product release kinetics of both the WT and mutant enzymes following the initial cycle of methylation (Fig. 5). The non-zero intercept or burst observed with WT EcoDam is consistent with a slow product release step that follows a much faster methyltransfer event. This slow step dominates the \( k_{\text{cat}} \) measured in steady state measurements, and the \( K_m \)app measured by the burst experiment. However, with the exception of the control R137A mutant, all mutants deficient in a direct contact to the flanking DNA phosphate backbone lost the burst seen with WT (Fig. 5). Thus, the rate-limiting catalytic step has changed from product release to a catalytic step up to and including methyltransfer (chemistry). This can happen in one of two ways. First, the rate of product release can be increased with respect to chemistry making the methyltransfer step the slowest step in the catalytic cycle. Second, the chemistry step can be slowed down in relation to product release so that it is the rate-dominating step. As seen in Fig. 5, the \( K_m \)app (derived from the slope of the curve) for the P substrate is faster for the flanking mutants when compared with that of the WT. Conversely, \( k_{\text{cat}} \)app for flanking mutants is decreased in relation to the WT for the N-P substrate. This suggests that mutation of a conserved phosphate interaction affects both the rate of product release and that of methyltransfer (chemistry). The exception to this generalization is the N126A mutant which, similar to the steady state results, was only able to detectably methylate the P substrate (Fig. 5A, inset). Like the other flanking mutants, N126A lost the burst, but the \( k_{\text{cat}} \)app for P decreased as opposed to the increase seen with the other mutants. Thus, we anticipate that if methylation were detectable on N-P, there would be no
ment of product release, whereas discrimination of a particular site by EcoDam involves the parti-
prior to dissociating from the DNA. We suggest that the dis-
highly processively when catalyzing multiple methyltransfers
The events up to and including chemistry; this is of particular
burst is a measure of chemistry. Additionally, our interest is in
WT EcoDam (black, dashed), R95A (red), N132A (light green), R116A (blue), K139A (pink), and N126A (dark green, inset). The WT shows a characteristic burst indicating that a step after methylation, most likely product release is rate-limiting. All of the conserved flanking phosphate interacting mutants have lost the burst and changed the rate-limiting catalytic step for EcoDam. The $k_{cat}$ for R95A, N132A, R116A, and K139A are increased up to 6-fold when compared with WT. N126A shows a 2-fold decrease in $k_{cat}$ when compared with WT. B, burst analysis on the non-preferred (N-P) substrate of WT EcoDam (black, inset), R95A (red), N132A (light green), R116A (blue), and K139A (pink, inset). No methylation on this substrate was observed for the N126A mutant under these conditions. All mutants have lost the characteristic burst seen with the WT enzyme as was seen on the P substrate. However, with the excep-
tion of K139A, the $k_{app}$ for the mutants are increased up to 60-fold when compared with WT. This is in direct contradiction to the relative increase in $k_{app}$ seen on the P substrate.
burst and the $k_{app}$ for this substrate would also be significantly slower than that of WT. Because the mutants change the rate-determining step of the methylation reaction, using $k_{cat}$ as a descriptor for site preference when comparing the WT and mutant enzymes is not necessarily accurate, being that $k_{cat}$ for WT EcoDam is a measurement of product release, whereas $k_{cat}$ for the mutants lacking a burst is a measure of chemistry. Additionally, our interest is in the events up to and including chemistry; this is of particular importance in the case of EcoDam where the enzyme behaves highly processively when catalyzing multiple methyltransfers prior to dissociating from the DNA. We suggest that the discrimination of a particular site by EcoDam involves the parti-
tioning between dissociation and catalysis, neither of which are directly observed in steady state measurements. We therefore tested the binding affinity of each mutant for cognate unmethylated GATC sites using polarization anisotropy. Interestingly, little to no affinity was lost on either the P or N-P substrates for each mutant (Table 2, Fig. 4).

To effectively measure the rate of methyltransfer for the WT and mutant enzymes, we designed single turnover experiments where the DNA is limiting and the enzyme and AdoMet are in excess (Table 2). The WT and control R137A single turnover measurements were preformed on a rapid quench instrument due to their high catalytic rates. All other single turnover experiments were completed by hand. The control R137A mutant and K139A showed only slight perturbations in $k_{chem}$ when compared with the WT enzyme; however, all other mutants showed a substantial decrease in $k_{chem}$ for the N-P substrate (200–2000-fold) and a slightly less, albeit still substantial decrease for the P substrate (15–150-fold). The most dramatic variation in the chemistry step of the catalytic cycle was seen with the alanine mutants of invariant residues Arg$^{95}$ and Asn$^{126}$. R95A showed decreases of 800- and 16-fold and N126A of 2000- and 150-fold for the N-P and P substrates, respectively. We suggest that these dramatic variations do not reveal themselves in the steady state $k_{cat}$ or $k_{m,app}$ mainly because the rate-limiting step in the catalytic cycle has been changed. For example, the $k_{cat}$, or rate of product release, for WT on P is 0.23 ± 0.09 min$^{-1}$ (Table 1), whereas the chemistry rate is substantially faster at 9 ± 2 min$^{-1}$ (Table 2). When comparing say, the R95A mutant $k_{cat}$ for P with that of WT EcoDam, the difference is only 2-fold. However, the $k_{cat}$ is dominated by chemistry in the case of R95A as is indicative of an enzyme that does not show burst kinetics. Although one would expect the $k_{cat}$ to be essentially equal to the $k_{chem}$ under these conditions, we suggest that different forms of the enzyme under single turnover (high enzyme, high DNA) and steady state (low enzyme, high DNA) conditions are affected differently by the phosphate mutants. Therefore, the real comparison is made when the mutant and WT enzyme are exposed to the same conditions to determine $k_{chem}$ (single turnover).

Our previous results suggested that WT EcoDam showed a 6-fold methylation preference ($E_{p}/E_{N,p}$) for the P substrate as opposed to the N-P substrate when considering the pre-steady state efficiency constants ($k_{chem}/K_{DNA}$) (25). This preference was mainly due to a ~10-fold decrease in the rate of chemistry on the N-P substrate when assayed at 4°C. Here we present evidence that at 22°C, the discrimination between sites $E_{p}/E_{N,p}$ for the WT enzyme is decreased to 2 (Table 2). We believe that many factors could be responsible for this affect. For example, although the enzyme is restricted to one methylation turnover, there are many conformational changes prior to catalysis, such as base flipping, which must occur prior to turnover. Each of these catalytic steps could have varying sensitivities to temperature, thus we see the greatest difference in $k_{chem}$ at 4°C. Additionally, the differences in the DNA structure at these two temperatures could have caused the decrease in methylation kinetics on the N-P substrate as A-tract DNA is bent at sharper angles at lower temperatures (30). The control R137A mutant and the conserved K139A mutant, like the
The Structural Basis of Processivity

WT enzyme, showed little to no methylation preference ($\frac{\xi_{P}}{\xi_{N-P}}$) when considering their pre-steady state efficiency constants for each substrate (Table 2). However, all other conserved mutants showed a dramatic increase in $\frac{\xi_{P}}{\xi_{N-P}}$ ranging from 32- to 89-fold for the P substrate. Again, the most dramatic change in $\frac{\xi_{P}}{\xi_{N-P}}$ came from the alanine mutants of invariant resides Arg95 and Asn126, which showed an 89- and 54-fold preference for the P substrate.

DISCUSSION

Processivity results from the partitioning of an enzyme on its polymeric (DNA) substrate between leaving (dissociating) and committing to doing catalysis at the next site. A processive enzyme favors the partitioning onto its substrate after catalysis, whereas a distributive enzyme preferentially dissociates. The EcoDam commitment term is itself made up of several components including dislocation from the methylated site, movement to nonspecific DNA, release/binding of cofactor, positioning at the next cognate site, tight binding of that site, and then catalysis (again). The ability of EcoDam to processively methylate multiple GATC sites on the same DNA substrate is dependent upon the DNA sequences immediately flanking the sites (14, 25). Here, we disrupted the enzyme-DNA interface with base pairs flanking the cognate site to better understand the structural basis for processivity and site preference. This work speaks to how nonspecific interactions between the protein and its DNA substrate are important for ensuring that processive catalysis is efficient, which requires the appropriate assembly of the active site. By mutating conserved residues that make contacts to phosphates outside the GATC recognition site, we have altered processivity whereas leaving intact the steady state efficiency constant, $\frac{k_{cat}}{K_{m}}$. Furthermore, although the residues are not obviously connected to the active site, changes in processivity and site preference occur through modulations in the chemistry constants, not DNA affinity.

Mutations of protein-DNA interfaces generally result in the loss of binding affinity or sequence discrimination resulting from an equal disruption of the ground (unbound) and transition (bound) states (34–37). However, the EcoDam mutants show minor changes in $K_{D}^{DNA}$, with changes in $k_{chem}$ being most dramatic (Table 2). Although less common, others have reported similar trends for DNA modifying enzymes (38–40). We suggest that two overlapping “uses” of the binding energy can be provided by the protein-DNA contact, which when disrupted, result in these two outcomes: changes in binding or changes in chemistry, or a blend of the two. If the contact contributes largely to tight binding or specificity, then its disruption results in affinity loss and/or loss of binding site discrimination (41). However, if the protein-DNA contact facilitates the correct assembly of the active site, its disruption will have minimal impacts on binding but will significantly alter catalysis.

For example, with the EcoRI endonuclease, base-specific contacts contribute up to $-10.3$ kcal/mol to the total free energy of binding energy, whereas each protein-phosphate interaction (nonspecific) contributes approximately $-1.3$ kcal/mol (41–43). In this case, the disruption of individual phosphate contacts from EcoRI to the DNA resulted in a range of binding perturbations (42). Jen-Jacobson (35) have referred to the situation wherein binding is left largely unaltered when a protein-DNA contact is altered as “structural adaptation.” In these situations, the enzyme energetically compensates for the loss of binding energy associated with a missing nonspecific contact, but in doing so loses the ability to reach the transition state. Alternatively, we suggest that if the binding energy is largely used to drive the correct assembly of the active site, removal of the binding energy is not anticipated to have a significant impact on binding, but rather will be observed as changes in the chemical step. It is the latter that we observe largely with the EcoDam mutants (Table 2, Fig. 4). Most recently, crystallographic analysis of WT HincII and a mutant deficient in an intercalation contact to the DNA flanking its cognate site confirmed the destabilization of the active site (39). Similar to what we observed for EcoDam phosphate mutants, the HincII intercalation mutant deficient in a flanking interaction showed no perturbation in $K_{D}^{DNA}$ values, but was drastically affected at the level of $k_{chem}$.

The positions of the residues shown in Fig. 1 in conjunction with the kinetic data described here provides direct evidence that a large, conserved protein-DNA interface plays a significant role in the processivity of an enzyme whose structure does not completely enclose the DNA. Methyltransferases share a set of 10 conserved motifs (I–X) that are responsible for AdoMet binding (I, II, III, and X) and catalysis (IV-VIII). The order of these motifs and their position relative to the variable TRD separate the methyltransferases into various classes (22, 23). For example, EcoDam belongs to the $\alpha$-class of methyltransferases with a motif arrangement of $X-I-II-TRD-III-IV-V-VI-VII-VIII$. The interior position of the TRD in relation to the AdoMet and catalytic motifs is unique to the $\alpha$ and $\beta$ classes of methyltransferases and suggests that proper folding and positioning of the TRD brings the two regions together in order for the methyltransfer to occur. The TRD of the $\alpha$-class of methyltransferases is further distinguished by the presence of a $\beta$-hairpin that contains various recognition elements (24). As shown in Figs. 1 and 2, the positions of the conserved residues that contact the phosphate backbone are dispersed within the TRD. The invariant residue Arg95 is located within an $\alpha$-helix adjacent to the AdoMet binding region, whereas the invariant Asn126 is located within the $\beta$-hairpin. Conserved residues Asn132, Lys139, and Arg116 are located in the loop regions flanking both sides of the $\beta$-hairpin.

Although none of the conserved or invariant residues mutated here are involved in the direct recognition of the GATC site, they do appear to anchor and stabilize the $\beta$-hairpin that contains the residues that recognize and intercalate into the cognate site. When considering the numerous crystal struc-
Our results suggest that stabilization of the TRD provided by the conserved residues that interact with the flanking DNA regulate the ability of the enzyme to read-out the flanking DNA and its ability to processively methylate multiple GATC sites. Restriction endonucleases such as EcoRI (26, 35, 46, 47) and EcoRV (40, 47) are responsive to DNA surrounding its cognate site, and a growing number of methyltransferases such as T4Dam (48) and Dmnt3a/Dmnt3b (49) have shown similar behavior. We present evidence that the evolutionarily conserved protein-DNA interface of EcoDam is responsible for the processive methylation of the ~20,000 GATC sites within the E. coli genome in addition to providing higher-order site discrimination based on the sequences and amount of DNA that surrounds a GATC site. The biological implications of having a mutant EcoDam deficient in processive catalysis are currently being pursued.

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The Structural Basis of Processivity

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