Nucleic Acids Research, 2005, Vol. 33, No. 10 **3235–3244**
doi:10.1093/nar/gki618

DNA bending by M.EcoKI methyltransferase is coupled to nucleotide flipping

Tsueu-Ju Su[1], Mark R. Tock[1], Stefan U. Egelhaaf[1,2], Wilson C. K. Poon[2] and David T. F. Dryden[1,*]

[1]School of Chemistry, The King's Buildings, The University of Edinburgh, Edinburgh EH9 3JJ, UK and
[2]School of Physics, The King's Buildings, The University of Edinburgh, Mayfield Road, Edinburgh EH9 3JZ, UK

Received March 21, 2005; Revised April 22, 2005; Accepted May 10, 2005

ABSTRACT

The maintenance methyltransferase M.EcoKI recognizes the bipartite DNA sequence 5′-AACNNNNNNNGTGC-3′, where N is any nucleotide. M.EcoKI preferentially methylates a sequence already containing a methylated adenine at or complementary to the underlined bases in the sequence. We find that the introduction of a single-stranded gap in the middle of the non-specific spacer, of up to 4 nt in length, does not reduce the binding affinity of M.EcoKI despite the removal of non-sequence-specific contacts between the protein and the DNA phosphate backbone. Surprisingly, binding affinity is enhanced in a manner predicted by simple polymer models of DNA flexibility. However, the activity of the enzyme declines to zero once the single-stranded region reaches 4 nt in length. This indicates that the recognition of methylation of the DNA is communicated between the two methylation targets not only through the protein structure but also through the DNA structure. Furthermore, methylation recognition requires base flipping in which the bases targeted for methylation are swung out of the DNA helix into the enzyme. By using 2-aminopurine fluorescence as the base flipping probe we find that, although flipping occurs for the intact duplex, no flipping is observed upon introduction of a gap. Our data and polymer model indicate that M.EcoKI bends the non-specific spacer and that the energy stored in a double-stranded bend is utilized to force or flip out the bases. This energy is not stored in gapped duplexes. In this way, M.EcoKI can determine the methylation status of two adenine bases separated by a considerable distance in double-stranded DNA and select the required enzymatic response.

INTRODUCTION

The EcoKI methyltransferase M.EcoKI, in common with all methyltransferases found in type I DNA restriction and modification (R/M) systems and many other DNA-binding proteins such as transcription factors and repressor proteins, binds to a defined bipartite DNA nucleotide sequence. M.EcoKI recognizes the specific double-stranded DNA target sequence 5′-AACNNNNNNGTGC-3′ and methylates the N6 position of adenine at and complementary to the underlined nucleotides within both parts of the target sequence. Any hexameric sequence can occupy the NNNNNN non-specific spacer within the target sequence. All type I R/M systems recognize bipartite targets with the adenine target bases 10 or 11 bases apart and separated by a non-specific spacer of 6 or 7 bases (1).

M.EcoKI displays a strong preference (50-fold difference between k_{cat}/K_M) for methylating hemimethylated targets, produced after each round of host DNA replication, over unmethylated targets (2,3). M.EcoKI is thus classified as a maintenance methyltransferase, in common with many eukaryotic methyltransferases, rather than the more common *de novo* methyltransferases of prokaryotic systems. Unmethylated targets are typically found in the DNA of phage and plasmids and trigger the restriction enzyme R.EcoKI to destroy the foreign DNA.

This preference of M.EcoKI for methylating hemimethylated target sequences requires a strong degree of internal communication between the parts of the enzyme surrounding each half of the bipartite target sequence. This communication of methylation status from one adenine target to the other presumably occurs via conformational changes induced by the recognition of the presence or absence of methyl groups on

*To whom correspondence should be addressed. Tel: +44 131 650 4735; Fax: +44 131 650 6453; Email: David.Dryden@ed.ac.uk
Present address:
Stefan U. Egelhaaf, Institut fur Physik der kondensierten Materie, Heinrich-Heine Universitat, 4025-Dusseldorf, Germany

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the adenines by the enzyme via steric clashes between the enzyme–S-adenosyl-methionine (SAM) cofactor complex and the DNA (4). It is likely that the methyl group on the cofactor is the sensor for the methyl group on the DNA, and it is highly probable that, in common with all other DNA methyltransferases (5), recognition of adenine methylation requires flipping of the target base out of the DNA helix into the enzyme methylation pocket, where it can interact with the cofactor (6).

Distinguishing the presence or absence of an adenine methyl group at two locations on the DNA sequence allows three responses by the enzyme, namely, recognition of doubly methylated targets, which promotes enzyme dissociation, recognition of hemimethylated targets, which promotes methylation to give doubly methylated targets, and recognition of completely unmethylated targets, which promotes restriction. To achieve this complex response to DNA methylation requires a large enzyme; M.EcoKI comprises two methylation (M) subunits and one specificity (S) subunit with a combined molecular weight of 169 000 Da (2). The addition of two further subunits forms the complete type I restriction endonuclease R.EcoKI, capable of performing both methylation and restriction. The footprint of M.EcoKI is ≥21 bp, so it can completely cover the long target sequence (7,8). The isolation of amino acid substitutions within the M subunit of EcoKI, which produced enzymes with *de novo* methyltransferase activity rather than maintenance methyltransferase activity, suggested that the communication process between the two methylation targets was primarily via the protein structure (9).

Bipartite DNA target sequences such as that recognized by M.EcoKI are commonly used recognition features for DNA-binding proteins. It is often found that the interaction between the protein and its DNA target is affected not only by the sequence flanking the recognition site, but also by the exact sequence of nucleotides in the middle of the target sequence, even though these are not formally recognized by the protein. Examples of such target sequences include those recognized by repressor proteins and transcription factors (10–20) and a subset of DNA R/M enzymes (21–23). Many studies on the binding properties of these proteins to DNA show that alteration in the DNA spacer sequence can alter binding affinity. This behaviour is generally attributed to a change in the flexibility of the spacer and a requirement for the protein to distort the DNA. In the case of proteins which distort their DNA site, it is often found that they bind preferentially to sequences which are more easily deformed to the correct conformation or inherently possess the correct distortion. It has also been found that the protein will bind with higher affinity to a more rigid DNA molecule than to a more flexible molecule if the average DNA conformation already matches that preferred for binding. This is a result of a smaller change in entropy upon binding to the stiffer DNA molecule. The introduction of single-strand nicks and gaps allows an assessment of the role of flexibility in binding. These complex interactions, involving changes in both the average static structure and the molecular dynamics, lead to complex thermodynamic behaviour of the enthalpy and entropy of binding (16,24–26).

In this paper we consider the role of the non-specific spacer nucleotides in the communication between the two adenine target sites in M.EcoKI. We find that these 6 bp, although not involved in sequence-specific DNA binding, play a crucial role in the DNA binding and activity of EcoKI.

MATERIALS AND METHODS

M.EcoKI was prepared as described previously (27). The oligonucleotide duplexes have the sequences shown in Table 1. Oligonucleotides (Eurogentec) were annealed by heating appropriate concentrations to 95°C in water followed by slow cooling overnight to room temperature. Ratios of DNA strands used were 1:1 for binding, 1.2:1 for 2-aminopurine (2AP) fluorescence and 2:1 for enzyme activity. The annealing efficiency of all duplexes or gapped duplexes (i.e. those with two short oligonucleotides annealed to a single long oligonucleotide) was checked using analytical high-performance liquid chromatography as described elsewhere (6). A molar ratio of 1:1 was obtained for the annealed duplexes and of 1:1:1 for the gapped duplexes, as required. Experiments were performed at least in triplicate at 295K for activity and 298K for other measurements. The reaction buffer

Table 1. Unmethylated oligonucleotide duplexes used in this study

Binding and enzyme activity	
NO-GAP	5′-label-TGTCTAGATATCGGCCT**AAC**CACGTG**GTGC**GTACGAGCTCAGGCG-3′ 3′-ACAGATCTATAGCCGGA**TTG**GTGCAC**CACG**CATGCTCGAGTCCGC-5′
1-GAP	5′-label-TGTCTAGATATCGGCCT**AAC**CACGTG**GTGC**GTACGAGCTCAGGCG-3′ 3′-ACAGATCTATAGCCGGA**TTG**GT CAC**CACG**CATGCTCGAGTCCGC-5′
4-GAP	5′-label-TGTCTAGATATCGGCCT**AAC**CACGTG**GTGC**GTACGAGCTCAGGCG-3′ 3′-ACAGATCTATAGCCGGA**TTG**G C**CACG**CATGCTCGAGTCCGC-5′
A-NO-GAP	5′-label-TGTCTAGATATCGGCCT**AAC**AAAAAA**GTGC**GTACGAGCTCAGGCG-3′ 3′-ACAGATCTATAGCCGGA**TTG**TTTTTT**CACG**CATGCTCGAGTCCGC-5′
A-4-GAP	5′-label-TGTCTAGATATCGGCCT**AAC**AAAAAA**GTGC**GTACGAGCTCAGGCG-3′ 3′-ACAGATCTATAGCCGGA**TTG**T T**CACG**CATGCTCGAGTCCGC-5′
2AP fluorescence	
NO-GAP-AP	5′-CACGGGCCT**APC**GATATC**GTGC**GTACGAGC-3′ 3′-GTGCCCGGA**TTG**CTATAG**CACG**CATGCTCG-5′
4-GAP-AP	5′-CACGGGCCT**APC**GATATC**GTGC**GTACGAGC-3′ 3′-GTGCCCGGA**TTG**C G**CACG**CATGCTCG-5′

The M.EcoKI target sequence is shown in bold. N6-methyl-adenine replaced the underlined adenines in some assays of enzyme activity and 2AP fluorescence. The 5′-label was hexachlorofluorescein for DNA binding and biotin for enzyme activity. P is 2AP.

was 20 mM Tris–HCl, pH 8, and 7 mM β-mercaptoethanol supplemented as required.

DNA binding was measured using a fluorescence anisotropy assay in which 5 nM hexachlorofluorescein-labelled DNA duplexes show an increase in anisotropy as protein binds to the DNA (8). All measurements were performed in buffer supplemented with SAM to 100 μM and with NaCl as required, as described elsewhere (8,28). Fitting was performed using Grafit (Erithacus Software) with either a model for single-site binding or a cooperative binding equation. The full single-site binding equation was used to take into account the tight binding between M.EcoKI and the DNA. The cooperative equation was used for the duplexes containing the A-tract spacer sequence, when binding was weaker and the assumption that the amount of unbound protein was equal to the total amount of protein was valid.

The fluorescence of 2AP when incorporated into DNA is a sensitive measure of base flipping by M.EcoKI (6). The fluorescence of 2AP from various DNA molecules was determined, as described previously (6), with 1 μM DNA, 2 μM M.EcoKI and 320 μM SAM in buffer supplemented with 50 mM NaCl and 0.1 mM EDTA. Excitation was at 320 nm with magic angle polarization.

M.EcoKI activity was measured by following the transfer of tritiated methyl groups from ^{3}H-methyl SAM (Amersham) to DNA which had been labelled on one 5′ end with biotin as described previously (6,29). The final concentration of other components in the buffer was 0.5 μM DNA, 0.5 μM M.EcoKI, 5 μM SAM and 50 ng/μl BSA. The SAM was a mixture of 10% ^{3}H-SAM and 90% non-radioactive SAM. In the 10 μl reaction volume there are 5 pmol of methyl targets in the hemimethylated DNA duplex and 10 pmol for the unmodified DNA duplex.

RESULTS

DNA binding

Figure 1 shows typical binding curves for the interaction between M.EcoKI and DNA duplexes using the fluorescence anisotropy assay. Binding to the A-4-GAP, NO-GAP and 4-GAP molecules was well described by a single-site binding equation and did not show cooperative binding behaviour at any NaCl concentration, indicating that sequence-specific interactions dominated under all experimental conditions for these duplexes (Table 2). In the case of A-NO-GAP and NaCl concentrations <50 mM, binding was not observed until large concentrations of M.EcoKI were present and the shape of the binding curve was indicative of some sort of cooperative binding process. This implied the binding of more than one copy of M.EcoKI to the duplex, a situation previously observed using gel shift assays at high protein concentrations (30). A cooperative binding equation for the binding of M.EcoKI to the DNA duplex was used to fit these experimental data. We do not believe this cooperative behaviour is of real physical significance; rather it reflects the difficulty in binding a single M.EcoKI molecule to DNA containing an inflexible A-tract sequence.

It is apparent from Figure 1 and the derived dissociation constants (Table 2) that the binding, in 0 or 25 mM NaCl, of M.EcoKI to duplexes containing a single-stranded region (4-GAP, A-4-GAP) was stronger than to the equivalent non-gapped duplexes (NO-GAP, A-NO-GAP). Binding of M.EcoKI to 1-GAP was identical to binding to NO-GAP within experimental error (data not shown). At these low

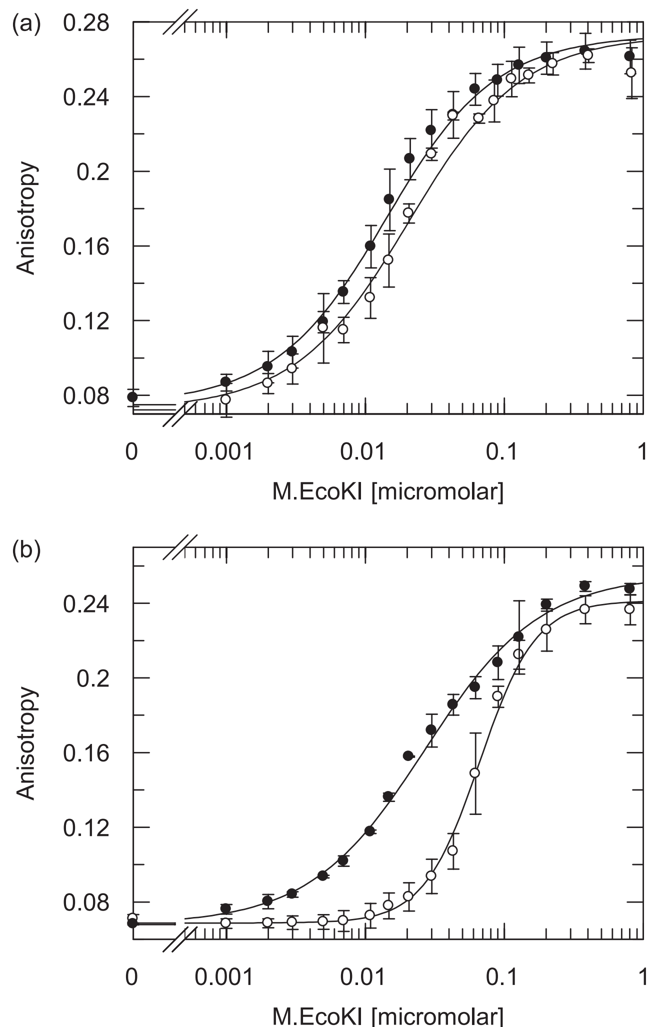

Figure 1. Titration of various hexachlorofluorescein-labelled DNA duplexes with M.EcoKI, in the absence of NaCl, increased the fluorescence anisotropy until all the DNA was bound. In each experiment the initial concentration of DNA was 5 nM and M.EcoKI was added to a final concentration of 1 μM. (**a**) NO-GAP DNA, open circles; 4-GAP DNA, filled circles. (**b**) A-NO-GAP DNA, open circles; A-4-GAP DNA, filled circles.

Table 2. Dissociation constants (nM) for binding of M.EcoKI to DNA determined in different concentrations of NaCl

DNA molecule	Sodium chloride concentration (mM)			
	0	25	50	100
NO-GAP	17.7 ± 2.2	51.0 ± 6.4	34.8 ± 5.3	77.8 ± 20.1
4-GAP	11.5 ± 1.0	22.9 ± 3.3	35.4 ± 2.8	90.7 ± 16.7
A-NO-GAP	68[a]	74[a]	80.2 ± 8.4	57.1 ± 8.3
A-4-GAP	24.3 ± 1.7	27.7 ± 3.1	25.1 ± 1.6	145.0 ± 19.8

[a]These values are determined from the concentration of M.EcoKI necessary to give a 50% change in the anisotropy signal. Cooperative fits to these data sets give dissociation constants of 4.9 ± 0.7 nM and 14.3 ± 4.8 nM with cooperativity values of 2.02 ± 0.04 and 1.65 ± 0.10 respectively.
Errors are standard deviation of the mean.

salt concentrations, the difference in binding affinity was small, ~1.5- to 2-fold between NO-GAP and 4-GAP. The difference was much greater between A-NO-GAP and A-4-GAP, with 50% binding being reached at ~2.7-fold lower M.EcoKI concentration for A-4-GAP, which reflects, we believe, the greater difficulty experienced by M.EcoKI in bending the stiffer A-NO-GAP duplex DNA. In this case, for simplicity we have compared the concentration of M.EcoKI required to reach 50% binding of the A-NO-GAP duplex with the dissociation constant for binding to A-4-GAP, as the binding to A-NO-GAP was clearly cooperative. However, when the NaCl concentration was increased, normal binding behaviour was restored, allowing a more valid comparison of the relative binding affinity of A-NO-GAP and A-4-GAP as described below.

The addition of NaCl reduced binding affinity as observed previously (8). However, the preference of M.EcoKI for binding to gapped DNA was maintained until the NaCl concentration reached 50 mM. The cooperativity observed with A-NO-GAP disappeared once the NaCl concentration reached 50 mM. At this salt concentration, the A-4-GAP duplex binds 3.2-fold better than the A-NO-GAP duplex. At 100 mM NaCl, dissociation constants for binding to NO-GAP, 4-GAP and A-NO-GAP were very similar, whereas A-4-GAP bound poorly. In other words, the enhanced binding of gapped DNA at low ionic strength is lost at high ionic strength. As discussed later, we attribute this to a greater degree of flexibility of the single-stranded region at high ionic strength resulting in a poor probability of M.EcoKI encountering both specific parts of its target sequence.

Methyltransferase activity

As expected from previous studies (2,29), methylation of duplex hemimethylated DNA target sequences was fast even for the DNA containing the A-tract spacer (Figure 2), and methylation of unmethylated target sequences was extremely slow, taking >1 h to achieve partial methylation of the target sequence (data not shown). It has previously been shown that the affinity of M.EcoKI for unmodified

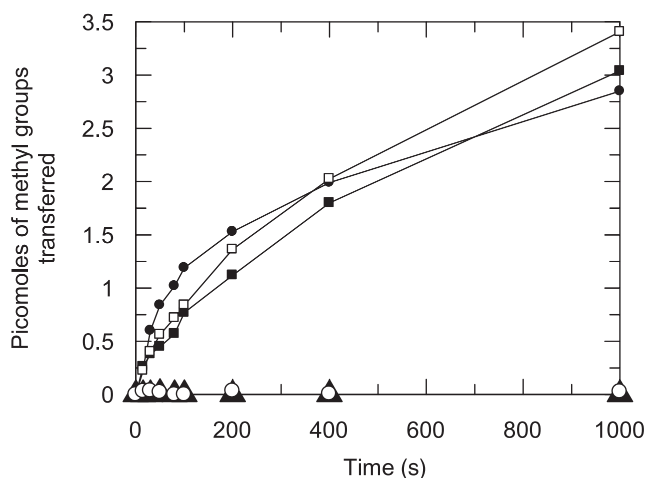

Figure 2. Transfer by M.EcoKI of ^{3}H-methyl groups from SAM to hemimethylated DNA duplexes. NO-GAP, filled circles; 1-GAP, filled squares; 4-GAP, filled triangles; A-NO-GAP, open squares; A-4-GAP, open circles.

and hemimethylated duplexes is the same within experimental error (7). It is reasonable to assume that this also holds for the gapped duplexes used in these assays as they have the same structure in the sequence-specific parts of the target sequence as the normal duplexes. The introduction of a single-strand gap of only 1 nt within the spacer sequence of a hemimethylated target (1-GAP) resulted in a reduction of ~25% in the initial rate of methylation compared with the normal duplex (NO-GAP), even though no detectable change in binding affinity was observed. However, in our experience, this assay is not particularly accurate (±20% on each data point), so we do not put any weight on this difference. Expanding the gap to 4 nt in either the 4-GAP or the A-4-GAP duplexes completely abolished methylation activity on hemimethylated DNA target sequences (and also on unmethylated target sequences, data not shown) despite the demonstrated ability of M.EcoKI to bind to these gapped DNA molecules.

Conformational changes

The complete abolition of methyltransferase activity by introducing the 4 nt gap was unexpected from the binding affinity experiment. Although it was possible that the chemical step of methyl group transfer was inhibited on the gapped DNA, it was more likely that the fault lay at an earlier stage in the reaction process somewhere between the initial DNA binding step, which we know occurs within manual mixing times (7), and the methyl group transfer step. After DNA and cofactor binding, the enzyme undergoes as yet undefined conformational changes showing enhanced sequence recognition (7) and changed footprint (8). Although these changes may be concomitant with the transfer of the methyl groups from the cofactor to the DNA, it is far more probable, as found for other methyltransferases (31–38), that these conformational changes occur prior to the methyl transfer.

For all characterized methyltransferases, the main conformational change occurring before methyl group transfer is base flipping in which the target base is swung by ~180° around the DNA phosphate backbone into the catalytic site of the enzyme (5). The fluorescence of 2AP when substituted for the methylatable base is often, but not always, an effective probe of this base flipping mechanism as the fluorescence is heavily quenched in a DNA duplex but the quenching is greatly reduced in the catalytic site of a base flipping enzyme (34,39–41). The equilibrium position of the 2AP is heavily weighted towards a base-paired, stacked, low-fluorescence location within the DNA double helix in the absence of a methyltransferase, and the equilibrium is shifted towards a base-flipped highly fluorescent position in the presence of the methyltransferase.

In our experiments, 2AP showed an enhanced fluorescence emission when placed at the methylation target sites within the target sequence in a complex of M.EcoKI with unmethylated NO-GAP-AP DNA (Figure 3a). Hemimethylated DNA spectra were identical to the unmethylated spectra (data not shown). The enhancement observed was ~7-fold at the emission maximum. Unusually, a further shift in the emission spectrum of 2AP occurred over a few minutes upon the addition of saturating amounts of SAM to the protein–NO-GAP-AP complex, as shown by spectrum (c) in Figure 3a. It has been postulated that this may be due to specific interactions of a photophysical

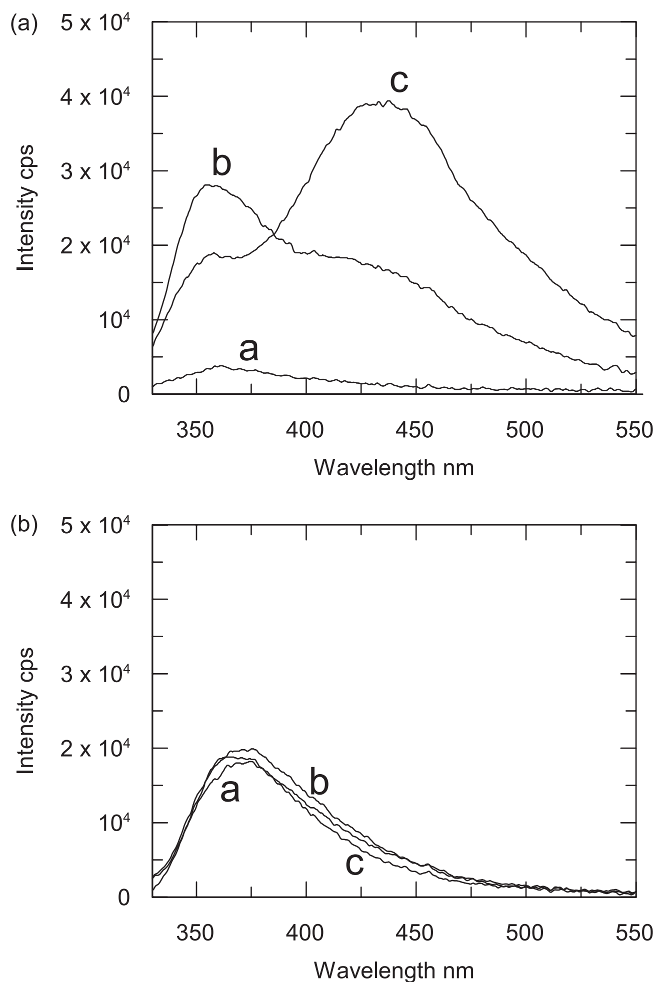

Figure 3. Emission spectra of 2AP in DNA duplexes. (**a**) Emission spectra of 2AP in unmethylated DNA duplexes (NO-GAP-AP). DNA duplex only, spectrum (a); DNA duplex with M.EcoKI, spectrum (b); DNA duplex with M.EcoKI and SAM taken after 15 min incubation with SAM, spectrum (c). Note that the intensity dramatically increases upon addition of M.EcoKI to DNA, and that there is a further increase with a red shift upon incubation with SAM. In these spectra, the weak buffer background and Raman scattering have been subtracted. (**b**) Emission spectra of 2AP in unmethylated, gapped DNA duplexes (4-GAP-AP). DNA duplex only, spectrum (a); DNA duplex with M.EcoKI, spectrum (b); DNA duplex with M.EcoKI and SAM taken after 15 min incubation with SAM, spectrum (c). In contrast to the normal duplex substrates in panel (a), the gapped substrates yield relatively unchanged spectra with M.EcoKI and SAM. In these spectra, the weak buffer background and Raman scattering have been subtracted.

nature between the 2AP and the active site of the M.EcoKI–SAM complex (6,42). This unusual fluorescence shift observed in a ternary complex of NO-GAP-AP DNA, SAM and M.EcoKI provides a highly distinctive signal for base flipping. Thus, although an atomic structure is not known for M.EcoKI or its complex with DNA, we assume from these results and previous data (6) that M.EcoKI can flip out the bases targeted for methylation either one at a time or both simultaneously.

The introduction of a 4 nt gap within the spacer sequence where the edges of the gap are separated by two intact base pairs from the 2AP probe had a dramatic effect on the emission of 2AP (Figure 3b). The emission of the 2AP in the 4-GAP-AP duplex was ~4 times greater than in the NO-GAP-AP duplex. This would suggest that the 2AP has more exposure to the solvent than in the normal duplex. As the 2AP is only 2 bp distant from the single-stranded spacer, perhaps the 2AP:T base pair is not as stable as in the normal duplex and transiently dissociates. This would be reminiscent of similar effects observed in duplexes were 2AP was placed near the end of a DNA duplex or at a mismatch (43–45). A transient 'fraying' or melting of the end would give an enhanced emission from the 2AP. Upon addition of M.EcoKI, the fluorescence enhancement produced by protein binding and base flipping was absent for the unmethylated 4-GAP-AP duplex (Figure 3b), and only 1.3-fold with the hemimethylated 4-GAP-AP duplex (data not shown). This negligible enhancement compared with NO-GAP-AP indicates that the equilibrium between stacked, quenched 2AP and unstacked, flipped 2AP in 4-GAP-AP was essentially unchanged by the binding of M.EcoKI. Furthermore, upon addition of SAM to these complexes of M.EcoKI with gapped DNA, no further change in 2AP fluorescence was observed with unmethylated 4-GAP-AP DNA and a small reduction in fluorescence was observed for hemimethylated 4-GAP-AP DNA (data not shown). There was no formation of the unusual, red-shifted emission observed with the normal NO-GAP-AP duplex. The absence of this distinctive signal for base flipping in ternary complexes of 4-GAP-AP DNA, SAM and M.EcoKI indicates that introducing a 4 nt gap has broken the mechanism used by M.EcoKI to flip adenine bases within its target sequence and explains the complete absence of DNA methylation despite the enhanced affinity for binding gapped duplexes.

DISCUSSION

The observation of enhanced binding of M.EcoKI to gapped DNA can be partially explained by the enhanced flexibility of the single-stranded region. This enhanced flexibility will allow the DNA to distort, via an induced fit mechanism, into the protein binding site with less expenditure of free energy. The effect is most dramatic when the non-specific spacer is composed entirely of A:T base pairs. The A-tract is known to be particularly inflexible and we observe very poor binding of M.EcoKI to DNA containing such an A-tract. Good binding is restored by introducing a gap to form a flexible single-stranded region. These results (Table 2) show that, if there are any contacts by M.EcoKI to the central 4 bp in the spacer region, they do not make any major contribution to binding affinity as their removal to create a gap does not diminish binding, but rather increases the binding affinity significantly. More specifically, we observed a weaker interaction between M.EcoKI and complete duplexes compared with gapped duplexes. This occurs despite the loss of potential non-sequence-specific interactions between the protein and the nucleotides removed to form the gap. In qualitative terms, the introduction of a 4 nt gap into the spacer sequence significantly changes the flexibility. The fact that a DNA molecule with increased flexibility shows stronger interaction with M.EcoKI than the corresponding complete duplex indicates that M.EcoKI must distort its DNA target. For our initial discussion we will assume that this distortion is due entirely to bending and we will subsequently consider other distortions such as twisting. Intuitively, it is clear that an intrinsically bent DNA duplex

whose bend happens to match that required for protein recognition will bind more strongly than a duplex which does not contain an appropriate bend. Furthermore, if two duplexes both have the correct bend but are of different flexibility, then the stiffer duplex will bind more strongly than the flexible duplex. However, if neither of the two duplexes has the correct intrinsic bend, it is easier for the more flexible duplex to bind to the protein because it is easier to distort the duplex to the correct conformation. (On the other hand, if the flexibility of the duplex is too great, then, for proteins such as M.EcoKI which bind to very long nucleotide sequences, the motion of the two target regions may be insufficiently correlated to allow the protein to make sufficient simultaneous contacts for binding to be efficient.) Similar arguments have been advanced (10) based upon extensive data for DNA binding by papilloma virus E2 proteins and for protein–ligand and protein–protein interactions (46,47). Data supporting these dynamic models for protein–ligand and protein–protein interactions are now receiving experimental support from e.g. NMR spectroscopy (48).

To make this discussion of our binding results more quantitative, we propose a simple, coarse-grained (i.e. no atomic details) model for the bending and binding of DNA to enzymes such as M.EcoKI. First we make estimates of the energetics of binding and bending. These are summarized in Figure 4. The interaction of (straight) DNA with M.EcoKI can be divided into two processes: binding of DNA (characterized by the dissociation constant K_{BI} in the case of a duplex and K_{bi} in the case of gapped DNA) and bending of DNA (characterized by K_{BE} and K_{be}, respectively). Experimentally we measure the dissociation constants K_1 and K_2 for the combined process of binding and bending of normal duplex and gapped duplex DNA, respectively. We can write

$$K_1 = K_{BI} \cdot K_{BE} = e^{(\Delta G_{BI}/kT)} \cdot e^{(\Delta G_{BE}/kT)}$$

where in the last step we used the relationship between a dissociation constant and the energy for binding a duplex, ΔG_{BI}, and bending the duplex, ΔG_{BE}. Corresponding equations hold for gapped DNA, with

$$K_2 = K_{bi} \cdot K_{be}.$$

Based on the finding that the introduction of a gap does not decrease the binding affinity, we assume that M.EcoKI does not interact with the DNA present in the nucleotide spacer. This would mean that the binding affinity of M.EcoKI is the same for duplex and gapped duplex, i.e. $K_{BI} = K_{bi}$, and thus $\Delta G_{BI} = \Delta G_{bi}$. Using this assumption we can relate the measured dissociation constants, K_1 and K_2 in Table 2, to the energy difference between bending a duplex, ΔG_{BE}, and bending a gapped duplex, ΔG_{be}, by the equation

$$K_1/K_2 = K_{BE}/K_{be} = e^{((\Delta G_{BI}-\Delta G_{be})/kT)} = e^{(\Delta\Delta G/kT)}.$$

Using the experimentally determined dissociation constants, we can calculate the energy difference $\Delta\Delta G$ between bending a duplex and bending a gapped duplex,

$$\Delta\Delta G = \Delta G_{BE} - \Delta G_{be} = kT\,ln(K_1/K_2).$$

In situations where the single-site binding equation adequately describes the data, we find, for salt concentrations of 0 and 25 mM, that $1.54 < K_1/K_2 < 2.20$ and hence 0.43 kT $< \Delta\Delta G < 0.79$ kT for NO-GAP compared with 4-GAP. For A-NO-GAP compared with A-4-GAP and a salt concentration of 50 mM, we find $K_1/K_2 = 3.2$ and hence $\Delta\Delta G = 1.16$ kT. These free-energy differences are about or just below the energy of thermal fluctuations.

Next, we seek to make sense of this estimate in terms of simple polymer physics. The polymeric aspects of DNA behaviour are often interpreted using the worm-like chain model (49–51). It describes DNA as a long, flexible cylinder with contour length L whose flexibility is described by the persistence length, l_p. The persistence length characterizes the distance over which DNA does not bend significantly and thus

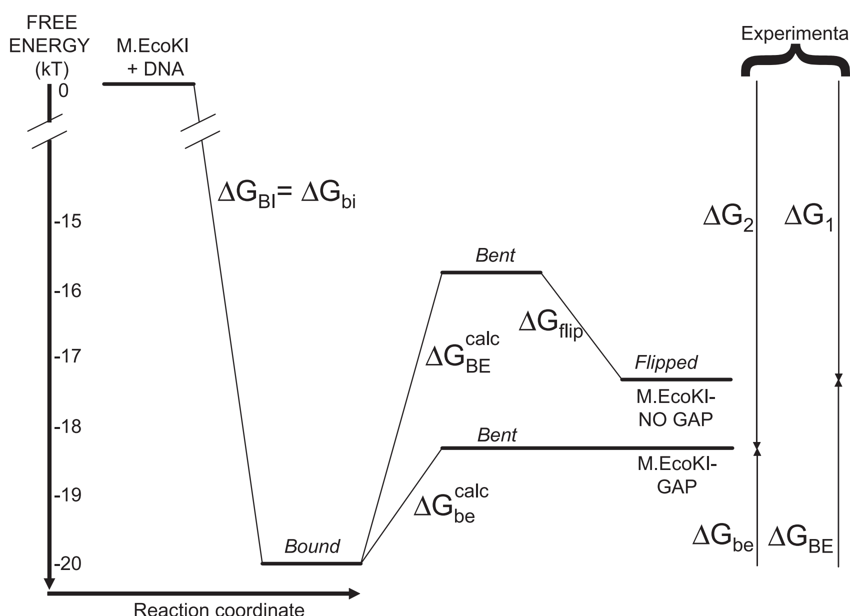

Figure 4. A thermodynamic sketch showing the proposed dissection of the experimental free-energy changes for binding M.EcoKI to duplex DNA (ΔG_1) or gapped DNA (ΔG_2) into steps for binding, bending and flipping as discussed in the text.

the orientation of the DNA is correlated over approximately this length. For double-stranded DNA the persistence length is very long [$l_{p1} \approx 50$ nm, which is equivalent to $\sim$150 bp; contour length per base pair L(bp) = 0.34 nm] and becomes longer, i.e. stiffer, at lower salt concentrations, although the salt dependence is weak (49). For the salt concentrations investigated here, the effect is $<\sim 3\%$ and we thus neglect the salt dependence of l_{p1}. Single-stranded DNA is much more flexible than double-stranded DNA and most estimates of its persistence length in high salt concentrations (51–54) give 0.7 nm $\leq l_{p2} \leq$ 3.0 nm for sequences containing pyrimidines, equivalent to only 1–4 nt (assuming a rise per nucleotide of 0.56 nm). This is consistent with the observation that gaps allow DNA to adopt a broad range of bend angles (51,55). The persistence length of single-stranded DNA, l_{p2}, shows a strong dependence on salt concentration, with a dramatic rise at low ionic strength, c_s (54):

$$l_{p2} = 0.642 + 0.4(c_s/M)^{-1/2} \text{ nm}.$$

This decrease in flexibility with decreasing ionic strength results not only from the decrease in electrostatic screening, but also from the well-known preference of nucleotide bases to stack upon each other in a loose, extended helical structure at lower ionic strength. While purine–pyrimidine or pyrimidine–purine steps have almost zero enthalpy of stacking, adenine–adenine steps have a considerable enthalpic component to stacking, 0.85 kT per step (56–58). This strong stacking with the adenine base results in a reduced flexibility and thus an increased persistence length: $\sim$8 nm for poly(dA) with an L(bp) per nucleotide of only 0.34 nm (52).

In the worm-like chain model, the energy ΔG_{BE}^{calc} needed for bending a duplex of contour length L by an angle Θ is

$$\Delta G_{BE}^{calc}/\text{kT} = \left(l_{p1}\Theta^2\right)/2L.$$

This relation is valid only for small L or large l_p, i.e. $L \ll l_p$, when the polymer does not assume a random configuration, which is satisfied for the 6 bp spacer considered here. In the absence of any detailed structural information, we further assume that the contour length, L, is 2.04 nm, equivalent to 6 bp, and that the bend angle is distributed evenly over each base pair within the spacer sequence irrespective of whether or not there is a single-stranded gap in the DNA. (These assumptions are expected to break down for small l_p, i.e. large salt concentrations, as discussed below.) For a total bend angle $\Theta = 33.6°$ and a 6 bp spacer, we obtain ΔG_{BE}^{calc} = 4.2 kT. [This bend angle is chosen because it is known that the complete EcoKI enzyme, of which M.EcoKI is the core, bends DNA and that this bend is similar in magnitude to that seen in the ocr protein, which binds very strongly to M.EcoKI (59,60). Since ocr contains a bend of 33.6° and apparently acts as a mimic of bent B-form DNA to inhibit DNA binding by M.EcoKI, it seems reasonable to propose that M.EcoKI will bend DNA by the same degree as that observed in ocr.] In the case of a gapped DNA spacer sequence, we have to consider three parts: a duplex of contour length $L_1/2$, a single-stranded region of contour length L_2 and again a duplex of contour length $L_1/2$. We assume that the DNA binding groove on M.EcoKI will constrain the conformation of the single-stranded spacer to essentially be identical to that of the normal double-stranded spacer. Hence, the contour lengths L_1 and L_2 (with $L_1 + L_2 = L$) are calculated based on L(bp) = 0.34 nm per base pair of double-stranded DNA or nucleotide of single-stranded DNA. The bend is distributed between the gapped and non-gapped part according to their contribution to the contour length, i.e. L_1/L and L_2/L. Hence, the bending energy is

$$G_{be}^{calc}/\text{kT} = \left(l_{p1}\Theta_1{}^2\right)/2L_1 + \left(l_{p2}\Theta_2{}^2\right)/2L_2$$
$$\text{with } \Theta_1 = \Theta(L_1/L) \text{ and } \Theta_2 = \Theta(L_2/L).$$

For $\Theta = 33.6°$ spread over the 6 base spacer sequence including the 4 nt gap, we obtain $\Delta G_{be}^{calc} \approx 1.6$ kT for the lowest salt concentrations covered in our experiments. Note that this model predicts ΔG_{be}^{calc} to be about equal to the thermal energy, kT, i.e. that a bend occurs spontaneously with a relatively high probability, quite in contrast to the bending of a non-gapped duplex, for which we predict ΔG_{BE}^{calc} = 4.2 kT. The energy difference between bending a duplex and bending a gapped duplex at low salt concentration is thus $\Delta\Delta G^{calc} = \Delta G_{BE}^{calc} - \Delta G_{be}^{calc} \approx 2.6$ kT.

The predicted value of $\Delta\Delta G^{calc} \approx 2.6$ kT can be compared with the range estimated from measured binding constants, 0.43 kT < $\Delta\Delta G$ < 1.16 kT. Considering the simplicity and lack of atomic details in our model, such better-than-an-order-of-magnitude agreement is noteworthy. It provides support for the contention that the observed preference for binding to gapped duplexes can be attributed mainly to the energy cost of bending the non-specific spacer region in the EcoKI target sequence. However, we should note that our experimental value for $\Delta\Delta$G also contains a term for base flipping in the duplex DNA which is absent for bending of the gapped DNA. In other words, the experimental K_{BE} is actually equal to $K_{BE}^{calc} \cdot K_{flip}$ and the experimental ΔG_{BE} is equal to $\Delta G_{BE}^{calc} + \Delta G_{flip}$, as shown in Figure 4. Thus the discrepancy between the calculated and experimental values of $\Delta\Delta G$ will actually be even smaller. Subtracting the experimental $\Delta\Delta G$ from the theoretical $\Delta\Delta G^{calc}$ suggests that the free energy of base flipping in the normal duplex is in the range 1.44 kT < ΔG_{flip} < 2.17 kT.

Our simple model cannot account for the observed salt dependence of DNA binding. In particular, upon increasing the salt concentration to 100 mM, the persistence length of single-stranded DNA becomes comparable to the length of the gap, so that the spacer is very flexible. We speculate that, under these conditions, M.EcoKI is less able to bind because the target sequences on the double-stranded regions on either side of the single-stranded gap can now fluctuate independently of each other. Thus, as expected, it is more advantageous to have continuous double-stranded DNA without gaps at physiological salinity.

Our model has considered only the role of DNA bending in binding affinity as other experiments strongly suggest this is occurring in the interaction of M.EcoKI with its DNA target (59,60). However, twisting of the non-specific spacer sequence may represent an alternative to bending. The torsional modulus for double-stranded DNA has been measured (61), but unfortunately a torsional modulus for single-stranded DNA does not appear to be available. However, it is certain to be much smaller than that for double-stranded DNA, and we assume it is negligible. From this one can calculate that to obtain a free-energy change ΔG_{twist} of the same magnitude as ΔG_{BE}^{calc}, i.e. 4.2 kT, a twist of 60° would be required within the

6 bp non-specific spacer and that $\Delta\Delta G^{calc}$ would be 4.2 kT as well, considerably larger than our experimental measurement and suggesting a larger value for ΔG_{flip}. This larger value of ΔG_{flip} would still be consistent with estimates of the free energy of base flipping as discussed below. Therefore, our results comparing M.EcoKI binding to duplex DNA or gapped DNA could also be interpreted in terms of twisting of the non-specific spacer instead of, or as well as, bending of the spacer. However, given the absence of any data addressing twisting for type I methyltransferases, we believe from previous results (59,60) that bending is the predominant effect.

The simplicity of our model means that it cannot be expected to offer a completely quantitative description of all aspects of our data for M.EcoKI. However, its very simplicity enables it to make a strong qualitative prediction for all cases of protein–DNA interaction in which the DNA target sequence contains a non-specific spacer region. If binding at low ionic strengths is enhanced by single-strand nicks or gaps in the spacer region, then the protein must be introducing a bend and/or twist into the DNA. If the binding becomes weaker, then the DNA either intrinsically has the correct bend/twist or the gap is too flexible. The latter possibility can be tested by reducing the ionic strength of the buffer to increase the persistence length of single-stranded DNA until it exceeds the length of the gap. Alternatively, the energetic costs predicted by the simple model may be masked by stronger, specific interactions between the protein and its DNA target (62).

The binding of the papilloma virus E2 protein to its DNA target appears to be the only comprehensively studied example of a protein binding to gapped DNA molecules [reviewed in (10)]. The recognition sequence for these proteins contains a 4 bp spacer sequence to which no contacts are made and a bend of between 40° and 50° is made in the DNA. In studies carried out in a high ionic strength buffer, these proteins preferentially bind to DNA predisposed by the nature of its sequence to contain a bend (20). The introduction of flexibility via single-strand nicks or gaps can enhance or weaken binding depending upon the particular viral protein used (11,13). The bovine virus protein prefers to bind to gapped DNA, whereas the human virus protein prefers to bind to double-stranded DNA which is stiff and contains the correct degree of bending. The introduction of single-strand breaks and two-base gaps gives experimental values of $0.29\ kT < \Delta\Delta G < 0.75\ kT$ for the bovine virus protein, similar to those we found in M.EcoKI.

Having discussed the coarse-grained physics of binding, we now turn to consider the specific effects of the single-stranded gap on the recognition of DNA methylation and enzyme activity by M.EcoKI.

Surprisingly, the relatively small thermodynamic and structural changes caused by the removal of the phosphate and other non-sequence-specific contacts with M.EcoKI have an enormous effect on enzyme activity, with an apparent total loss of methyltransferase activity for the 4-base-gap DNA. The introduction of a gap into the non-specific spacer is clearly preventing the enzyme–DNA complex from forming an active transition state complex. Normally the enzyme prefers to methylate hemimethylated DNA, and it was assumed, since mutant proteins without this preference could be isolated (9), that the communication of the methylation state at one site to the other site occurred via the protein structure (4,63). A methylated adenine when flipped out into the active site would clash with the methyl group on the cofactor SAM. This poor fit of flipped methyl-A, if it could occur at all, would alter the protein conformation over a long enough range to reach the other methylation site and induce methylation. If neither adenine were methylated, both would fit into the methylation sites, but the absence of any steric effect would not position the adenines correctly for methyltransferase activity. However, as activity is abolished by introducing a single-strand gap into the non-specific spacer sequence, whereas binding is maintained or even enhanced, communication of methylation status clearly goes through the DNA as well as through the protein.

Some form of structural and/or dynamic information-transfer mechanism must pass through the 8 bp intervening between the two methylation target bases, a distance of 2.72 nm in B-form DNA. Our calculations using the worm-like chain model and the structure of the DNA mimic protein ocr (59,60) strongly suggest that M.EcoKI bends the DNA within the non-specific spacer part of its target sequence. Additional support for a gross DNA structural distortion comes from small angle X-ray scattering, circular dichroism and footprinting studies performed on a similar type I methyltransferase, M.EcoR124I (64,65). Kneale and colleagues showed not only that the protein conformation changed upon DNA binding but that a large change in the DNA duplex, as determined by circular dichroism and DNA footprinting, also occurred irrespective of the methylation state of the DNA target sequence. Although an exact interpretation of the spectroscopic change could not be made, they suggested that a delocalized twisting over a considerable number of base pairs would result in a large change in the circular dichroism signal. This long-range distortion would be additional to the localized distortion around the methylation sites clearly visible in the DNA footprinting. Unpublished data mentioned by Kneale (64) showed that M.EcoR124I did not appear to bend the DNA substrate. However, more recent atomic force microscopy data show that M.EcoR124I does bend its DNA substrate by angles ranging broadly between 0° and 60° (66). Therefore, the bending analysis presented for M.EcoKI may be equally applicable to other type I methyltransferases.

We suggest that bending of the non-specific spacer in the target sequence can be used as a way of communicating between the two methylation sites. We can postulate that the bases at the focus of the bend in the non-specific spacer will be compressed and unfavourable steric clashes will result unless this compression is relieved. Our results show that flipping of 2AP at the methylation sites occurs with duplex DNA targets but not with gapped duplexes. If the bases in the non-specific spacer are indeed compressed in duplex DNA, it would appear that the DNA relieves this stress by actively flipping the methylation target bases out of the helix into the catalytic pockets in M.EcoKI. If little compression is generated by the bending of the DNA upon binding of M.EcoKI, as is apparently the case for the gapped duplexes, then there is no free energy to be gained by flipping out the methylation target bases and the 2AP fluorescence is unchanged (Figure 4). Therefore, we suggest that when M.EcoKI binds, the energy cost of bending the DNA is reduced by forcing the methylation target bases out of the DNA helix—an indirect but active method to drive base flipping.

There has been much discussion about the driving force for base flipping, with different enzymes appearing to use passive

or active mechanisms (31–36,67,68). The passive mechanisms rely upon the natural fluctuations in base pairs, which, on the 10–100 ms timescale, actually break apart to expose individual bases projecting at least partially out of the helix. As most methyltransferase reactions also occur on this timescale, the enzyme essentially waits for a base pair to break and then captures it in the catalytic site. Active mechanisms expend energy to push or pull the required base out of the helix. Whatever the actual mechanism, calculations show that the energy required for base flipping is fairly small, being several times the thermal energy kT at room temperature (69–73), a value in the range of ΔG_{flip} determined by comparison of our experimental $\Delta\Delta G$ with $\Delta\Delta G^{calc}$.

CONCLUSION

Our results and calculations show that if binding affinity improves when a gap is introduced into the DNA then DNA bending (and/or twisting) must be occurring. This is a general result for any DNA-binding protein. If no improvement in affinity is observed then the distortion may still be occurring but the effect is being masked by the free energy of specific interactions between the protein and the DNA. In the case of M.EcoKI binding to DNA, evidence suggests that bending rather than twisting is being introduced into the DNA and that this bend is localized within the non-specific spacer within the recognition sequence. We postulate that this would cause a compression of the DNA bases. The steric problems introduced by the bend are reduced by the flipping out of the adenine bases targeted for methylation by the enzyme. Hence DNA bending is used by M.EcoKI to cause base flipping and allows communication of the methylation status of the two adenines along the length of the DNA.

ACKNOWLEDGEMENTS

W.C.K.P. and D.T.F.D. thank the EPSRC for funding T.J.S. D.T.F.D. thanks the BBSRC for funding M.R.T. We are grateful to Miss Emma Kemp for assistance in early experiments. We thank an anonymous referee for useful comments about DNA twisting. Funding to pay the Open Access publication charges for this article was provided by JISC.

Conflict of interest statement. None declared.

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