Nucleotide flipping by restriction enzymes analyzed by 2-aminopurine steady-state fluorescence

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ABSTRACT

Many DNA modification and repair enzymes require access to DNA bases and therefore flip nucleotides. Restriction endonucleases (REases) hydrolyze the phosphodiester backbone within or in the vicinity of the target recognition site and do not require base extrusion for the sequence readout and catalysis. Therefore, the observation of extrahelical nucleotides in a co-crystal of REase Ecl18kI with the cognate sequence, CCNGG, was unexpected. It turned out that Ecl18kI reads directly only the CCGG sequence and skips the unspecified N nucleotides, flipping them out from the helix. Sequence and structure conservation predict nucleotide flipping also for the complexes of PspGI and EcoRII with their target DNAs (CCWGG), but data in solution are limited and indirect. Here, we demonstrate that Ecl18kI, the C-terminal domain of EcoRII (EcoRII-C) and PspGI enhance the fluorescence of 2-aminopurines (2-AP) placed at the centers of their recognition sequences. The fluorescence increase is largest for PspGI, intermediate for EcoRII-C and smallest for Ecl18kI, probably reflecting the differences in the hydrophobicity of the binding pockets within the protein. Omitting divalent metal cations and mutation of the binding pocket tryptophan to alanine strongly increase the 2-AP signal in the Ecl18kI–DNA complex. Together, our data provide the first direct evidence that Ecl18kI, EcoRII-C and PspGI flip nucleotides in solution.

INTRODUCTION

Base or nucleotide flipping is the displacement of a base in regular B-DNA from the helix into an extrahelical position. First observed by X-ray crystallography for the bacterial C5-cytosine methyltransferases M.HhaI (1) and M.HaeIII (2), nucleotide flipping (base extrusion) has been documented later for other methyltransferases (3–5), glycosylases (6–9), glycosyltransferases (10,11) and various DNA repair enzymes (12–17). Some enzymes, e.g. the methyltransferases, flip a nucleotide of only one DNA strand (1–5). Others, like endonuclease IV, alter the backbone conformations of both strands flipping the deoxyribose and nucleotide at an abasic site (13). Either way, nucleotide flips occur because enzymes need access to a DNA base to perform chemistry. For example, DNA methyltransferases transfer the methyl group to the extruded base, while glycosylases involved in DNA repair excise the extrahelical base (18). Typically, an amino acid side chain is intercalated into the DNA to fill in the ‘hole’ introduced after the base flipping event (6,12,19).

Nucleotide flipping in the co-crystals of restriction endonuclease Ecl18kI with cognate DNA came as a surprise (20). In a functional sense, Ecl18kI is a ‘standard’ Type II restriction endonuclease (REase): it recognizes pentanucleotide sequence CCNGG and cuts phosphodiester bonds on the 5' sides of the outer cytosines to generate 5' overhangs (21). Although the endonuclease does not subject the central bases to any kind of modification, in the crystal structure these bases were clearly extrahelical and accommodated in pockets on Ecl18kI made by the side chain atoms of Arg57 on one face and the indole ring of Trp61 on the other face (Figure 1). Unlike in other complexes with flipped nucleotides, there was no ‘hole’ in the DNA and no amino acid intercalation. Instead, the DNA was compressed, so that the base pairs adjacent to the flipped nucleotides stacked directly against each other. The resulting DNA compression reduced the length of the interrupted 5 bp stretch CCNGG to the length of a 4 bp stretch CCGG and made the distance between the scissile phosphates in the Ecl18kI–DNA complex equal to the distance between the scissile phosphates in the

Figure 1. Flipped nucleotides in the Ecl18kI–DNA complex structure (2FQZ). (A) General view of the Ecl18kI dimer–DNA complex. Protein is shown in spacefill. Residues 60–69 and 91–136 are removed for clarity. DNA is depicted in red. (B) Binding ‘pocket’ for the flipped out base. A flipped adenine base is accommodated in the ‘pocket’ made by the side chain atoms of Arg57 on one face and the indole ring of Trp61 on the other face.

NgOMIV complex with a continuous sequence GCCGGC (20,22). Therefore, we suggested that Ecl18kI uses base flipping to adapt the conserved sequence readout machinery for the interrupted target site and predicted that the evolutionary related REases EcoRII and PspGI that cut the related CCWGG sequence before the first C, might also flip nucleotides (23–25).

Nucleotide flipping in solution by Ecl18kI, PspGI and EcoRII remains to be established. So far, it is only supported by the observation that PspGI accelerates deamination of the central cytosine in the incorrect GCCGG sequence, which differs from the canonical sequence at the center (26). 2-Aminopurine (2-AP) has often been used as a fluorescence probe to detect base flipping in solution (27–35). The 2-AP fluorescence is highly quenched in polynucleotides due to the stacking interactions with neighboring bases (36) and therefore increases strongly when the base is flipped out of the DNA helix (28,29). Here, we use 2-AP as a fluorescence probe for base flipping and provide the first direct evidence in solution that Ecl18kI, the C-terminal domain of EcoRII (EcoRII-C) and PspGI extrude the central base pair while interacting with their recognition sites.

MATERIALS AND METHODS

Oligonucleotides

2-AP containing oligodeoxynucleotides were obtained from Integrated DNA Technologies (HPLC grade, Coralville, USA), non-modified oligodeoxynucleotides were from Metabion (HPLC grade, Martinsried, Germany). In order to assemble oligoduplexes, appropriate oligodeoxynucleotides (Table 1) containing 2-AP or non-fluorescent control strands were mixed with a 1.05-fold molecular excess of complementary strands in the reaction buffer A (33 mM Tris-acetate, pH 7.9 at 25°C, 66 mM potassium acetate), heated to 85°C and allowed to cool slowly over several hours to room temperature. For the DNA binding and cleavage studies one strand of the 25 bp duplexes was 5’-end labeled with [γ-33P]ATP (Hartmann Analytic, Braunschweig, Germany) using a DNA labeling kit (Fermentas, Vilnius, Lithuania).

Proteins

The wt Ecl18kI, Ecl18kI mutant W61A, EcoRII-C, PspGI and MvaI proteins were purified and concentrations were determined by measuring absorbance at 280 nm as described in (24,25,37,38). All protein concentrations are indicated in terms of the dimer, except for MvaI, which is a monomer in solution (38).

Mutagenesis

The W61A mutant of Ecl18kI was obtained by the modified QuickChange Mutagenesis Protocol (39). Plasmid pET21b(+)_R.Ecl18kI [ApR] (20) was amplified by PCR using Pfu DNA polymerase (Fermentas, Vilnius, Lithuania) and two complementary (partially overlapping) primers obtained from Metabion (desalt grade, Martinsried, Germany) containing the desired mutation. After PCR the methylated parental (non-mutated) plasmid was digested with DpnI (Fermentas, Vilnius, Lithuania). Escherichia coli BL21(DE3) cells carrying the plasmids pVH1 [KnR] (with lacIq) and pHSG415ts [CmR] bearing the ecl18kI/M recognition site were transformed with the PCR product by the CaCl2 method. Plasmid DNA was isolated by the alkaline lysis procedure and purified using the GeneJET™ Plasmid Miniprep Kit (Fermentas, Vilnius, Lithuania). Sequencing of the entire gene of the mutant confirmed that only the designed mutation had been introduced.

Gel mobility shift assay

Gel shift analysis of DNA binding by wt proteins and Ecl18kI W61A mutant protein was performed by titrating 32P-labeled 25 bp oligoduplex (see Table 1) at 0.1 nM concentration with increasing amounts of protein. Kd values were evaluated as described elsewhere (40).

DNA cleavage activity

The DNA cleavage activities of wt Ecl18kI, Ecl18kI mutant W61A, EcoRII-C and PspGI were monitored using a 25 bp oligoduplex containing a 32P-label either in the top or the bottom DNA strand (Table 1). Cleavage rates of both strands were evaluated separately. Ecl18kI cleavage reactions were conducted at 20°C in the reaction buffer A, containing 10 mM MgCl2 and 0.1 mg/ml BSA.

Table 1. Oligoduplexes used in this study*

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Oligoduplex</th>
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<tr>
<td>5’CGCACOCCTCCTGGAAAGGACACTA 3’</td>
<td>Oligoduplex I</td>
</tr>
<tr>
<td>3’GGCTGCGGAAAGGACACTA 5’</td>
<td>Oligoduplex II</td>
</tr>
<tr>
<td>5’CGCACOCCTCCTGGAAAGGACACTA 3’</td>
<td>Oligoduplex II</td>
</tr>
<tr>
<td>3’GGCTGCGGAAAGGACACTA 5’</td>
<td>Oligoduplex III</td>
</tr>
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*Ecl18kI/EcoRII-C/PspGI/MvaI recognition site is in boldface; 2-aminopurine, is in boldface and underlined.

Table 1. Oligoduplexes used in this study*
using 200 nM of oligoduplex and 300 nM of protein. EcoRII-C and PspGI cleavage reactions were performed in the same buffer A at 25°C using 200 nM of oligoduplex and 1000 nM of protein. Aliquots were removed at timed intervals and quenched by mixing with loading dye [95% (v/v) formamide, 0.01% (w/v) bromphenol blue, 25 mM EDTA] before denaturing gel electrophoresis. The samples were analyzed and quantified as described in (41).

Fluorescence spectroscopy

All fluorescence measurements were acquired in photon counting mode on a Fluoromax-3 (Jobin Yvon, Stanmore, UK) spectrofluorometer equipped with Xe lamp. Sample temperatures were maintained at 25°C by a circulating water bath. Oligoduplexes I or II were used as 2-AP labeled DNA (Table 1). Emission spectra (340–420 nm) were recorded at an excitation wavelength $\lambda_{ex} = 320$ nm with excitation and emission bandwidths of 2 and 8 nm, respectively. At least two scans were averaged for each spectrum. Sample emission spectra were collected in reaction buffer A in the presence and absence of calcium acetate on 250 nM DNA alone or 250 nM DNA mixed with a 5-fold excess of the protein to ensure saturation of the fluorescence signal. Control spectra used for the background subtraction corrections were collected under identical conditions except that oligoduplex III was used instead of the fluorescent DNA. The fluorescence emission value of the corrected spectrum was determined at the emission maximum (see Supplementary Table S1) for each sample. For the oligoduplex titration experiment, emission spectra of the 250 nM oligoduplex I with protein in a 0–2000 nM range were collected.

RESULTS

Probes for Ecl18kI triggered nucleotide flipping

We have used the 2-AP fluorescence assay to monitor base flipping in the Ecl18kI–DNA complex in solution. A number of 25 nt oligoduplexes that contain the fluorescent base analog 2-AP were designed (Table 1). In the oligoduplex I, 2-AP was incorporated within the CCNGG sequence instead of A in the central base position. In the oligoduplex II, 2-AP was introduced immediately adjacent to the target site. Like most restriction enzymes, Ecl18kI requires Mg$^{2+}$ ions for DNA cleavage. In the absence of divalent cations, it forms a rather weak complex with cognate DNA (Supplementary Table S2). Addition of Ca$^{2+}$ ions that do not support cleavage significantly increased Ecl18kI–DNA complex stability (24). Gel shift experiments revealed that 2-AP incorporation into the target sequence had no effect on the affinity of Ecl18kI for cognate DNA in the presence of Ca$^{2+}$ ions (Figure 2). In the buffer supplemented with Mg$^{2+}$ ions, Ecl18kI cleaved 2-AP containing and lacking oligoduplexes at identical rates (data not shown).

Figure 2. Gel mobility shift analysis of the interactions between Ecl18kI and oligoduplexes. (A) Ecl18kI binding of the oligoduplex III containing the recognition sequence. (B) Ecl18kI binding of the cognate oligoduplex I containing 2-AP instead of the central A base. The binding reactions contained $^{33}$P-labeled 25 bp oligoduplex (0.1 nM) and the Ecl18kI at concentrations as indicated by each lane. Samples were analyzed by PAGE under non-denaturing conditions (see Material and Methods Section). Gels were run in the presence of 5 mM of Ca$^{2+}$ ions.

Ecl18kI in the presence of Ca$^{2+}$ ions

We titrated the 2-AP containing oligonucleotides with Ecl18kI in the binding buffer supplemented with Ca$^{2+}$ ions and monitored the change of the 2-AP fluorescence intensity at 367 nm (Figure 3A, Supplementary Table S1). The free oligoduplex containing 2-AP at the central position showed low signal because the fluorescence was quenched due to base stacking interactions (Figure 3B). When Ecl18kI bound at saturating concentrations to oligoduplex I, which contains 2-AP in the central position, the fluorescence intensity increased 6.5-fold. In contrast, only small changes were observed with oligoduplex II, which carries 2-AP outside of the target site (Figure 3B and C). The change of fluorescence intensity for the oligoduplex I suggests that the 2-AP stacking with DNA bases is disrupted. It is compatible with nucleotide flipping, which has been shown to enhance 2-AP fluorescence to varying extents in different systems (27-35).

Ecl18kI in the absence of Ca$^{2+}$ ions

Gel shift analysis showed highly decreased binding of Ecl18kI to cognate DNA in the absence of Ca$^{2+}$ ions (see, Supplementary Figure S1). However, we found that at much higher enzyme and DNA concentrations used in the fluorescence titration experiments, Ecl18kI formed a binary complex with cognate DNA in the absence of Ca$^{2+}$ ions (Figure 3D). The $K_d$ value obtained from the titration data was $52 \pm 12$ nM. In the Ca$^{2+}$-free buffer, Ecl18kI binding to the oligoduplex I containing the 2-AP in the central position increased the fluorescence intensity ~28.5-fold at saturating protein concentrations, while only small changes were observed with oligoduplex II (Figure 3E and F). The 2-AP signal in the buffer without
Ca\(^{2+}\) ions was \(\sim 4\) times higher than the signal in the buffer supplemented with Ca\(^{2+}\) (Figure 3D). These results suggest that the structure of the complex formed in the presence of Ca\(^{2+}\) ions may differ from that formed without Ca\(^{2+}\).

**Ecl18kI W61A mutation**

2-AP fluorescence is often quenched in the hydrophobic environment of a protein (30,31,42,43). In the crystallographic complex of Ecl18kI with DNA, the flipped nucleotides are accommodated in pockets that are lined by tryptophan Trp61. In order to test whether Trp61 quenches 2-AP fluorescence, we replaced this residue with alanine. The Ecl18kI W61A variant did not cleave cognate oligoduplex III or the 2-AP containing oligoduplex I radioactively labeled at either strand, but it retained the ability to bind both oligoduplexes albeit at \(\sim 10\)-fold decreased affinity according to the gel shift assay (see, Supplementary Table S2). Binding of Ecl18kI W61A to oligoduplex I in the presence of Ca\(^{2+}\) ions increased the 2-AP fluorescence intensity \(\sim 125\)-fold (Figure 4) suggesting that the mutant was able to flip out the central nucleotide. 2-AP fluorescence in the ternary W61A–DNA–Ca\(^{2+}\) complex was \(\sim 20\) times higher than in the wt Ecl18kI–DNA–Ca\(^{2+}\) complex (Figure 4). Thus, the W61A mutant data support the assumption that low 2-AP fluorescence in the ternary complex with the wt protein is due to the quenching of the extruded base by stacking interactions with the Trp61 residue. However, one cannot exclude that increased space in the binding pocket of the W61A mutant may allow a different orientation of the extrahelical 2-AP and affect the fluorescence intensity.

**EcoRII-C and PspGI**

The C-terminal domain of the EcoRII restriction enzyme and the PspGI restriction enzyme are specific for the CCWGG sequence (where W stands for A or T) and cleave it before the first C. It was suggested that
Ecl18kI and EcoRII-C/PspGI may be evolutionarily related (23–25). This raises the intriguing question of whether EcoRII-C and PspGI also flip the central W nucleotides while interacting with their target sites. The EcoRII structure, which was solved in the absence of DNA (44) shows a very similar fold to Ecl18kI (20) except that it has an extra N-terminal regulatory domain (Figure 5). Structural comparison between Ecl18kI and the C-terminal domain of EcoRII reveals that Arg57 and Trp61, which sandwich the flipped bases in the Ecl18kI–DNA cocrystal structure, spatially coincide with the Arg222 and Tyr226 of EcoRII suggesting that EcoRII may flip the central base similarly to Ecl18kI (20). Therefore, we analyzed base flipping by EcoRII-C in solution using the 2-AP fluorescence assay. EcoRII-C turned out to bind to the oligoduplex I containing the 2-AP in the central position (see, Supplementary Table S2) and binding was accompanied by a 12-fold increase of fluorescence (Figure 6), suggesting that the base is extruded from the double helix.

The structure of the PspGI enzyme which recognizes the same CCWGG sequence as EcoRII but lacks the extra N-terminal domain is not yet known, but modeling studies (26) suggest significant similarities to Ecl18kI (Figure 5). Moreover, genetic studies support the PspGI model and provide indirect evidence that PspGI may flip central nucleotides within a sequence that matches its target site except for the presence of a G-C pair instead of the A-T pair at the center (26). We found that PspGI binding is not sensitive to the modification, hence 2-AP is a good surrogate for A in experiments with these enzymes.

Evidence for Ecl18kI-triggered nucleotide flipping

2-AP fluorescence is strongly quenched if the base is stacked in DNA and increases when the stacking is perturbed. Therefore, 2-AP fluorescence does not necessarily indicate nucleotide flipping, since it could also be attributed to a less drastic DNA unstacking deformation.

DISCUSSION

Enzymes typically flip nucleotides to gain access to otherwise poorly accessible bases. Based on crystallographic information and sequence analysis, we have suggested that the restriction endonucleases Ecl18kI, EcoRII and PspGI employ base flipping in a novel way to achieve specificity for their targets and to adjust their cleavage patterns.

MvaI

Recently, we have solved the crystal structure of MvaI restriction enzyme that recognizes the CC/WGG sequence identical to that recognized by EcoRII and PspGI but cleaves it before the W nucleotide as indicated by the ‘/’ (38). In the MvaI–DNA complex structure, the DNA conformation does not deviate essentially from the canonical B-form and there is no evidence for base flipping. Binding studies in solution revealed that MvaI binds 2-AP-containing oligonucleotide I (see, Supplementary Table S2), however, this did not lead to an increase of 2-AP fluorescence (Figure 6).

2-AP as a probe

Here, we used 2-AP fluorescence as a probe to monitor base flipping by Ecl18kI, EcoRII-C and PspGI in solution. Fluorescence probes to monitor nucleotide flipping in solution have to balance the conflicting requirements for mimicry of natural nucleotides and environment-sensitive fluorescence in a wavelength range not obscured by background signal from the other nucleotides and protein. 2-AP represents a good compromise in this respect. At neutral pH it makes a Watson–Crick base pair with T, which is only slightly weaker than the natural A-T pair (45). We have found that Ecl18kI, EcoRII-C and PspGI binding is not sensitive to the modification, hence 2-AP is a good surrogate for A in experiments with these enzymes.
However, the much higher 2-AP fluorescence increase in the W61A–DNA–Ca\(^{2+}\) ternary complex compared to the wt–DNA–Ca\(^{2+}\) ternary complex (Figure 4) strongly suggests that in the latter complex the fluorophore comes close to the indole ring of Trp61 for efficient quenching. Moreover, the lack of activity of the Ecl18kI W61A mutant in the presence of Mg\(^{2+}\) ions and the nearly 10-fold reduced affinity to DNA are also consistent with a loss of interactions between the flipped nucleotide and the indole ring of Trp61.

In contrast to the effect of the W61A mutation, which can be readily attributed to the different hydrophobicities of tryptophan and alanine, the effect of Ca\(^{2+}\) ions on the 2-AP fluorescence is more difficult to interpret. According to the gel shift assay, the binary Ecl18kI–DNA complex is much weaker than the ternary Ecl18kI–DNA–Ca\(^{2+}\) complex (Supplementary Table S2). Nevertheless, 2-AP fluorescence in the binary complex is much higher than in the ternary complex in presence of Ca\(^{2+}\) ions (Figure 3). Moreover, the fluorescence increase in the binary complexes of wt Ecl18kI and W61A mutant is comparable (data not shown). Perhaps the flipped bases are firmly trapped in the quenching pockets of the enzyme in the presence of Ca\(^{2+}\) ions, but retain mobility and therefore higher fluorescence in the absence of Ca\(^{2+}\) ions?

### Evidence for EcoRII and PspGI-triggered nucleotide flipping

Ecl18kI and EcoRII/PspGI are evolutionarily related and recognize target sequences that differ only in the central base pair. The strong 2-AP fluorescence increase upon addition of EcoRII-C and PspGI supports the idea that these enzymes also flip the central nucleotides of their target sequences. The 2-AP fluorescence intensity differences (Figure 6) likely reflect the nature of the enzyme pockets that accommodate the flipped bases. A structure-based alignment indicates that these pockets are lined by Trp61 in Ecl18kI, Tyr226 in EcoRII and Phe64 in PspGI. In the absence of crystal structures of DNA complexes of EcoRII and PspGI, it remains unclear whether the differences in 2-AP fluorescence in the enzyme–DNA complexes are purely due to different hydrophobicities, or whether changes in the orientation of the aromatic side chains or other alterations around the flipped nucleotides contribute to the observed effects.

Unlike Ecl18kI, which accepts any base pair at the center of its recognition sequence, EcoRII and PspGI cleave only target sequences with a central A-T pair. Modeling argues against the possibility that discrimination against a G-C pair could be due to the base-specific hydrogen bonding interactions in the EcoRII/PspGI DNA complexes. Instead, the strength of the hydrogen bonding interaction of the central base pair may determine specificity. Cytosine deamination experiments, however, provide indirect evidence that PspGI flips the central nucleotides in the sequence CCCGG, which is not efficiently cleaved by PspGI (26). As rates for flipping and back-flipping are not yet known, it is conceivable that the detailed balance between these two processes decides which substrates are cleaved by Ecl18kI, EcoRII and PspGI.

### SUMMARY

The results of the 2-AP fluorescence assay provide direct evidence that Ecl18kI, EcoRII and PspGI unstack bases at the center of their recognition sequences and flip them into pockets that are formed by the enzymes (20). The new results show that prior findings from the co-crystal structure of Ecl18kI with DNA are relevant in solution. Moreover, the new results complement genetic experiments on PspGI, which had provided indirect evidence that this restriction enzyme flips the central cytosine in the sequence CCCGG, which is related to the PspGI target sequence, but not cleaved by PspGI. Finally, the change of the 2-AP fluorescence signal upon Ecl18kI/EcoRII-C/PspGI binding paves the way for stopped flow experiments to measure base flipping in real time (27,30,31,33–35,46–50) and time resolved fluorescence studies (43,51) to identify possible intermediates on the base flipping pathway.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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