DNA looping and translocation provide an optimal cleavage mechanism for the type III restriction enzymes

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***EcoP15I* is a type III restriction enzyme that requires two recognition sites in a defined orientation separated by up to 3.5 kbp to efficiently cleave DNA. The mechanism through which site-bound *EcoP15I* enzymes communicate between the two sites is unclear. Here, we use atomic force microscopy to study *EcoP15I*–DNA pre-cleavage complexes. From the number and size distribution of loops formed, we conclude that the loops observed do not result from translocation, but are instead formed by a contact between site-bound *EcoP15I* and a nonspecific region of DNA. This conclusion is confirmed by a theoretical polymer model. It is further shown that translocation must play some role, because when translocation is blocked by a Lac repressor protein, DNA cleavage is similarly blocked. On the basis of these results, we present a model for restriction by type III restriction enzymes and highlight the similarities between this and other classes of restriction enzymes.**

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Introduction

Enzymes that hydrolyse ATP to move on a DNA molecule are essential in all organisms. ATP hydrolysis is coupled to a series of protein conformational changes to facilitate the movement of the protein in the correct direction on the DNA. These proteins have been grouped into several structural superfamilies and contain amino-acid ‘DEAD-box’ and associated motifs (Delagoutte and Von Hippel, 2002, 2003; Singleton and Wigley, 2002). From an operational viewpoint, they can be classified into two main groups. DNA helicases separate the DNA strands as they move along the DNA,

whereas DNA translocases move on the double helix without separating the strands. In this second group, one finds the chromatin-remodelling factors and the complex type I and type III restriction and modification (R/M) enzymes.

The complex R/M enzymes require the interaction between two copies of the enzyme bound to specific recognition sites on the same DNA molecule (Dryden *et al.*, 2001; Bourniquel and Bickle, 2002). These recognition sites can be thousands of basepairs (bp) apart and it is believed that highly processive translocation of these huge distances is required to bring the two enzymes into collision with each other. Collision then triggers DNA cleavage at the collision point for type I R/M enzymes, but at a fixed point near to the original recognition site for the type III R/M enzymes. The observation of loops of DNA being produced during translocation indicates that these restriction enzymes remain bound to their recognition sequences during translocation (Yuan *et al.*, 1980; Endlich and Linn, 1985; Ellis *et al.*, 1999; Berge *et al.*, 2000; Reich *et al.*, 2004). The operation of these restriction enzymes therefore appears to require location of the specific recognition sequence by diffusional searching of the DNA molecule (Halford and Marko, 2004), followed by a further, ATP-driven collision of the DNA-bound proteins.

The translocation and collision model for type I R/M enzymes is based upon many experiments, including direct imaging using electron microscopy and atomic force microscopy (AFM) (Yuan *et al.*, 1980; Ellis *et al.*, 1999; Berge *et al.*, 2000; Janscak and Bickle, 2000), cleavage assays (Studier and Bandyopadhyay, 1988), time- and location-dependent displacement of DNA-bound probes (McClelland *et al.*, 2005; Stanley *et al.*, 2006), *in vivo* measurements (Davies *et al.*, 1999; Garcia and Molineux, 1999) and, most persuasively, studies using magnetic tweezers (Seidel *et al.*, 2004; Stanley *et al.*, 2006). Translocation requires the enzyme to remain at its target site and to reel in the DNA from both sides of the site, thereby producing expanding DNA loops. Stalling of translocation by collision with another type I R/M enzyme, leads to DNA cleavage at the collision site. Translocation requires ATP hydrolysis by the DEAD-box motifs present in the restriction subunits of the type I R/M enzyme (Dryden *et al.*, 2001; Bourniquel and Bickle, 2002; Sistla and Rao, 2004).

Given that restriction by both type I and type III R/M enzymes requires ATP hydrolysis and cooperation between distant sites, it was postulated that the type III R/M enzymes cooperate via ATP hydrolysis-driven one-dimensional (1D) DNA translocation and collision (Meisel *et al.*, 1995). The type III DNA R/M enzymes are multifunctional oligomeric enzymes widely distributed in bacteria and archaea (Sistla and Rao, 2004). For *EcoP15I*, cleavage occurs 25 bases downstream on the top strand and 27 bases downstream on the bottom strand from the first adenine in the target sequence, 5′-CAGCAG-3′. In addition, type III R/M enzymes show a very

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strong preference for cleavage to occur only when there are two target sites on the same piece of DNA oriented towards each other in a head-to-head fashion (Meisel *et al*, 1992, 1995; Raghavendra and Rao, 2004). When two sites face each other, an enzyme bound at each site cooperates with the other bound enzyme to cut 3' to only one of the two target sites. The sites can be separated by ~20–3500 bp and the cooperation and cleavage at only one site still applies. The cooperation and DNA cleavage is blocked or reduced if the sites are in the wrong orientation (tail-to-tail or head-to-tail) (Meisel *et al*, 1995), if there is only one site on the DNA (Raghavendra and Rao, 2004), if a Lac-repressor protein molecule is present on the DNA between the two head-to-head sites (Meisel *et al*, 1995; Raghavendra and Rao, 2004), or if the sites are on catenated DNA with only one site per catenane ring (Peakman and Szczelkun, 2004). These results were proposed to rule out cooperation between distant type III R/M enzymes via three-dimensional (3D) looping of the DNA (Janscak *et al*, 2001; Peakman and Szczelkun, 2004).

The type I R/M enzymes, in common with other DNA translocases and helicases (Delagoutte and Von Hippel, 2002, 2003) require hydrolysis of approximately one molecule of ATP per bp of DNA moved. Measurements on several type I R/M enzymes have given values ranging from 3 to 0.1 ATP per bp (Eskin and Linn, 1972a,b; Endlich and Linn, 1985; Davies *et al*, 1998, 1999; Bianco and Hurley, 2005). In contrast, ATP consumption by type III R/M enzymes, although certainly required for DNA cleavage, occurs at a much slower rate—only 1% of that used by type I R/M enzymes (Meisel *et al*, 1995; Sears *et al*, 2005). For example, *PstII*, travels 82 bp per ATP molecule hydrolysed (Sears *et al*, 2005). This negligible rate of ATP hydrolysis is open to different interpretations. First, the type III R/M enzymes may be the most energy-efficient DNA translocases known, capable of translocating many bp per ATP molecule hydrolysed. Alternatively, the type III R/M enzymes may be normal DNA translocases and hardly move any distance at all on the DNA substrate; however, if this were the case, one could not then explain the cooperation between sites separated by thousands of bp (Peakman and Szczelkun, 2004; Sears *et al*, 2005).

Here, we use AFM to show that 3D DNA looping is used by the type III R/M enzyme *EcoP15I* as the major mode of communication between distant sites. We also reconcile our data with earlier work that provided evidence both for and against looping by these enzymes. The size of the loops observed is described well by the polymer theory of semiflexible long-chain molecules. However, cleavage can still be blocked by intervening proteins on the DNA, indicating that translocation is also required. We propose that for two DNA-bound enzymes to communicate between two distal sites and then to trigger cleavage only when the sites are in a suitable orientation enzyme dimerisation, looping and limited directional translocation driven by ATP-hydrolysis are required. In this way, the type III R/M enzymes appear to have found the optimal strategy for their function.

Results

Complexes in the presence of nonhydrolysable ATP γ S

EcoP15I-DNA complexes were formed in a high-salt buffer in the presence of the ATP analogue, ATP γ S, on template SS,

Figure 1 *EcoP15I*-DNA complexes in the presence of ATP γ S. (A) Complexes on single-site template SS in high-salt buffer. Scale bars, 500 nm in main image and 250 nm in montage images. (B) Histogram showing the position of the *EcoP15I* relative to the nearest DNA end.

which has one recognition site close to one end. Under these conditions, binding of ATP γ S to *EcoP15I* can be shown by the decrease in tryptophan fluorescence intensity at 331 nm when increasing amounts of the analogue are added (data not shown). However, *EcoP15I* is unable to cleave DNA in the presence of ATP γ S (Mucke *et al*, 2001). Therefore, *EcoP15I* is able to bind, but not hydrolyse, ATP γ S. In AFM images, enzymes bound to the DNA were observed as globular features located very close to one end of the DNA contour (Figure 1). Analysis of these complexes shows the majority of enzymes were bound at the recognition site (Figure 1B). This experiment indicated that the enzyme bound site-specifically before any process relying on ATP hydrolysis.

The presence of ATP on a single-site template

Pre-cleavage complexes were formed on template SS in the presence of ATP, both in the high-salt buffer used in the previous experiment, and also in a low-salt buffer. *EcoP15I* exhibits optimum cleavage activity in the high salt buffer, when provided with a suitable DNA template (i.e., one containing two head-to-head sites), and reduced activity in the low-salt buffer. The effect of ATP on the behaviour of *EcoP15I* on a single-site template was striking (Figure 2): loops were observed in both low- and high-salt buffers in contrast to the result in the presence of nonhydrolysable ATP γ S (compare Figures 1A and 2A for the high-salt buffer). *EcoP15I* was able to form loops in low-salt buffer (Figure 2B), suggesting that the final step of DNA cleavage was reduced by the low ionic strength, rather than site recognition or any prior ATP-dependent process such as looping. Previous

Figure 2 *EcoP151*-DNA complexes in the presence of ATP on one site template. (A, B) Complexes on single site template SS in high-salt (A) and low-salt (B) buffer. Scale bars, 500 nm in main image and 250 nm in montage images. (C-F) Histograms showing the contour length of DNA loops formed by *EcoP151* in the presence of ATP (C), the number of loops formed by complexes exhibiting looping (D), position of the *EcoP151* and loop origin relative to the nearest DNA end (E) and the loop apex angle (F).

studies have shown that *EcoP151* activity is preserved in low-salt buffers (Raghavendra and Rao, 2004) and for $[\text{Na}^+]$ in the range 0–2 M (Bist *et al*, 2001).

The length of the loop, or ‘loop contour’, showed an asymmetric, narrow distribution, centred around 200 nm, equivalent to four persistence lengths of double-stranded DNA (Figure 2C). In low-salt buffer, the majority of complexes appeared to form loops, and of those that looped the majority formed only one loop; in the high-salt buffer two loops per complex were observed more frequently (Figure 2D). The infrequency of multiple loop formation may be due to the DNA region upstream of the recognition sequence not being sufficiently long to form stable loops. The increased looping in the high-salt buffer may be due to an increased DNA-enzyme interaction in this buffer.

The single-site template used in this experiment supports cleavage at much lower rates than a two-site head-to-head template, thus allowing observation of pre-cleavage complexes by AFM in either buffer (Raghavendra and Rao, 2004). However, in subsequent experiments, on templates with two sites the low-salt buffer is essential, as the high-salt buffer would result in rapid cleavage and the absence of complexes in AFM images. Furthermore, high-salt buffers reduce the image quality due to adsorbed salt layers on the mica surface. It is possible that the 5 mM KCl in the low-salt buffer may have promoted nonspecific looping distinct from the ATP-dependent looping reported above. However, this was not the case, as the high ionic strength of the high-salt buffer did not abolish the formation of loops; instead the number of loops per complex increased (Figure 2D). Further,

the loop contour distribution, the enzyme position and loop apex angle distributions (discussed below) all appeared similar (Figure 2C–E).

When the position of the enzyme on the DNA template was measured, it was found that not all complexes remained at their recognition sites close to the end (Figure 2E); a second population appeared closer to the centre of the DNA molecule. This may reflect the dynamic nature of looping. If the enzymes were to form a succession of loops with a nonspecific region of DNA then in a number of cases, the enzyme might relinquish its specific, rather than nonspecific contact. This would result in the enzyme being repositioned on the DNA contour. The angle subtended by the loops as they enter the enzyme, the ‘loop apex angle’, was also quantified (Figure 2F). This parameter is used in comparing our data to theoretical models described below.

The presence of ATP on two-site templates

The experiment above was repeated in low-salt buffer using template HH (Figure 3A and B) containing two head-to-head sites separated by 3816 bp, with 525 and 653 bp between each site and the nearer DNA end. These distances are sufficient to allow looping of DNA both up- and downstream of the recognition site (in contrast to template SS where looping is physically constrained by the template structure). This head-

to-head arrangement is known to support efficient DNA cleavage (Janskak *et al*, 2001). Complexes formed on this template showed highly looped structures (Figure 3B and D). The distribution of the loop contour (Figure 3C) was similar to that of template SS. The observed complexes exhibited one, two or no free DNA ends or ‘arms’. In cases where at least one DNA arm was observed, this was measured and, although the distribution was broad, two peaks could be identified at 140 nm (411 bp) and 180 nm (529 bp), (Figure 3E). This is slightly less than the 525 and 653 bp expected and may indicate that the DNA is partially dehydrated from B to A form on the mica surface, as observed previously (Rivetti and Codeluppi, 2001).

The orientation of the site at 525 bp was then switched to produce template HT with the sites in a head-to-tail orientation. If DNA translocation were responsible for the observed loops then the head-to-tail orientation would lead to little collision and stalling and hence minimal observation of loops. Furthermore, loops with different loop contours from template HH would be observed. Alternatively, if looping, rather than translocation were responsible for the observed geometry, then the relative orientation of the two sites should have little effect on the appearance of complexes and loop contours. The complexes observed in the head-to-head and head-to-tail case look similar (Figure 3A and B), and the loop contour distributions appeared identical (Figure 3C),

Figure 3 *EcoP15I*-DNA complexes in the presence of ATP on two-site template. (A) Complexes on two-site template HH and derivatives, in low-salt buffer. Scale bars, 500 nm in main image and 250 nm in montage images. (B) Montage of looped complexes on template HH and HT. (C–E) Histograms showing the contour length of DNA loops formed by *EcoP15I* on template HH and HT (C), the number of loops formed by complexes exhibiting looping on template HH and –+ (D), position of the *EcoP15I* and loop origin relative to the nearest DNA end on template HH (E).

Figure 4 The Lac repressor blocks DNA cleavage by *EcoP15I*. (A) Lac-DNA complexes on template HH in Lac buffer. (B) *EcoP15I*-DNA complexes in Lac buffer in the presence of ATP on template HH. (C) *EcoP15I*-Lac-DNA complexes in Lac buffer in the presence of ATP on template HH. Scale bar = 500 nm. (D) Lac repressor assay on plasmid pTYB1. The five *EcoP15I* sites are marked on the map as filled arrows and the Lac-binding sites as rectangles. M, molecular weight marker; lane 1, DNA + ATP; lane 2, DNA + ATP + *EcoP15I*; lane 3, DNA + ATP + *EcoP15I* + Lac; lane 4, DNA + ATP + *EcoP15I* + Lac + IPTG; lane 5, DNA + ATP + *EcoP15I* + Lac (2 × concentration).

reinforcing our hypothesis that loops are formed by 3D looping rather than translocation.

For control experiments (Figure 3A), either one (template -+) site or both sites (template --) were deleted. On template -- a small amount of nonspecific binding was observed; however, no DNA looping was observed, indicating that a recognition site is required for looping. On template -+, DNA looping was observed with predominantly two or three loops. This is an increase from the situation with

template SS where single loops predominated in the low-salt buffer (Figure 2D). Template --+ has a longer sequence up- and downstream of the recognition sequence that would favour the formation of two loops.

One further observation of *EcoP15I* on two-site substrates is that invariably only one protein complex is observed on the looped DNA (Figures 3 and 4). This complex often appears greater in volume than the complex observed on a single-site DNA substrate (Figures 1 and 2). Given the complexity of the

looping patterns and the influence of AFM tip convolution effects, an accurate assessment of molecular volume is unfeasible. However, we propose that the presence of two sites on a DNA substrate leads to a dimerisation of *EcoP15I*, in the same way as observed with the structurally related type I R/M enzyme, *EcoKI* (Ellis *et al*, 1999). This dimerisation process is likely to be fast as looping rapidly brings distant segments of DNA, with or without bound protein, into collisional contact (Rippe *et al*, 1995; Rippe, 2001).

Presence of Lac repressor

The Lac repressor has been shown to prevent DNA cleavage by *EcoP15I* when the Lac-binding site was placed between two head-to-head *EcoP15I*-recognition sites. It was assumed that the Lac repressor acted to block translocation, preventing the enzymes from associating and hence cleaving (Meisel *et al*, 1995; Raghavendra and Rao, 2004). Here, we have used template HH, which has a Lac-binding site positioned between the two head-to-head oriented *EcoP15I* sites (2102 and 1714 bp away from each respective site; see Figure 4). We formed complexes in buffer that promotes Lac repressor binding and also supports moderate *EcoP15I* activity (Meisel *et al*, 1995). This corresponds to $2 \times$ low-salt buffer used in the above experiments (i.e., 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂ and 10 mM KCl). Under these conditions, the Lac repressor bound with a degree of nonspecificity and could form loops on its own (Figure 4A). This was expected as the repressor not only binds with high affinity to its operator sequence, but also to similar pseudo-site sequences with a reduced affinity (Riggs *et al*, 1972). It is possible that these loops may prevent further looping due to the action of *EcoP15I*, thus causing a block of cleavage through a different mechanism from that previously proposed (Meisel *et al*, 1995; Raghavendra and Rao, 2004). Formation of *EcoP15I* complexes in Lac buffer led to fewer looped structures than in low-salt buffer (compare Figure 4B with Figure 3A), presumably because, under these conditions, some complexes were able to undergo cleavage and dissociate from the template. When repressor was present in the solution, *EcoP15I* was still able to form loops (Figure 4C), although it is not clear whether these looped complexes represented complexes in which cleavage was blocked by a Lac repressor.

The same experiment was repeated on plasmid pTYB1. This has five *EcoP15I* sites (Figure 4D, a–e), two pairs of which are oriented head-to-head facilitating cleavage at any one of these four head-to-head sites. One Lac-binding site is positioned between the a–e pair of head-to-head sites, 2102 bp from one site and 1714 bp from the other. The c–d pair of sites are separated by only 116 bp; therefore, fragments resulting from cleavage at either of the c or d sites will differ by a length too subtle to be detected by the assay. *EcoP15I* digested the plasmid into four fragments corresponding to cleavage first to full-length linear (FLL), and then secondary cleavage of FLL at either of the remaining sites (lane 2). The efficiency of the secondary cleavage of FLL appeared reduced, as seen by the presence of undigested FLL. As cleavage at site c or d cannot be distinguished, only four rather than eight fragments are observed. Furthermore, two of these fragments migrated as one band at 5.6 kbp. When Lac repressor was present, secondary cleavage of the FLL DNA was much reduced, suggesting that cleavage of the a–e pair, which contains the Lac-binding site, was reduced (lane 3). When

IPTG was added to the reaction before addition of *EcoP15I*, the DNA–Lac repressor binding was abolished and the cleavage resembled that in the absence of Lac repressor, (lane 4). Elimination of cleavage specifically, and solely, of the a–e pair could not be achieved by increasing the Lac repressor concentration. Instead, a twofold increase in the Lac repressor concentration resulted in plasmid DNA, indicating a total elimination of cleavage even at the c–d pair that does not contain a Lac-binding site (lane 5). Assuming that the Lac repressor acted as a block to translocation only, this result showed that *EcoP15I* underwent translocation at some step during the cleavage process. The presence of residual cleavage activity indicates that the Lac repressor may occasionally be knocked off by a translocating *EcoP15I*, or that 3D looping is able to sometimes circumvent the bound Lac repressor. Limited translocation coupled with 3D looping would explain the extremely low rates of ATP hydrolysis observed for *EcoP15I*.

Comparison of loop contour to polymer physics models

The formation of loops depends critically on the length of the loop relative to the stiffness (persistence length) of the DNA and various models have been proposed (Yamakawa and Stockmayer, 1972; Hagerman and Ramadevi, 1990; Rippe *et al*, 1995; Rippe, 2001; Sankararaman and Marko, 2005). These models consider the DNA as a freely jointed chain or as a worm-like semiflexible chain, with the loops being circular, tear-drop-shaped or kinked. For loops which do not contain sharp kinks in the DNA, a tear-drop shaped loop is more energetically favourable than a circular loop. These theories make predictions about the size of loops to be found in DNA. The most probable loop size is ~ 500 bp with shorter loops being much less probable and longer loops decreasing slowly in probability as a function of $n^{-3/2}$, where n is the number of bp. These theories have been confirmed by experiments (Shore *et al*, 1981; Shore and Baldwin, 1983; Hagerman and Ramadevi, 1990; Sankararaman and Marko, 2005).

The measured DNA loop contours from Figures 2 and 3 on templates SS, HT and HH were pooled with earlier results on pUC19 plasmid DNA (data not shown), together with published work on a linear 2691 bp fragment containing two head-to-head sites (Reich *et al*, 2004). These data were plotted on a normalised histogram (Figure 5) and compared to the tear-drop model with an acute angle at the apex held by the enzyme. Assuming the enzyme does not constrain this angle, an energy minimum is predicted at 51.2°. This compares well with our experimental value measured in Figure 2. The theoretical model fits remarkably well with the experimental data, with significant deviation only occurring at large loop contours. This deviation is probably due to the fact that we use finite length DNA templates in which long loops will be under represented. It is clear that the loop sizes observed by AFM for *EcoP15I* are well described by the tear-drop model and that there is no requirement for translocation.

Discussion

Our data suggest both a structural model for the type III R/M enzymes, and a functional reason for the unusual site orientation required for the cleavage reaction, which has implications for the operation of all restriction enzymes requiring two target sites for cleavage.

Figure 5 Comparison of loop contour length with a polymer physics model. Histogram represents data for templates SS, HT and HH, pUC19 plasmid DNA and the published data of Reich *et al* (2004). The line shows the probability distribution for the tear-drop model scaled (vertically) to match the histogram of experimental data. (Yamakawa and Stockmayer, 1972; Sankararaman and Marko, 2005).

Structural implications for *EcoP15I* and other type III restriction enzymes

The results on a single-site template indicate that *EcoP15I* has three DNA-binding sites, one for sequence recognition and two for the formation of DNA loops. The *EcoP15I* enzyme exists as a Mod_2Res_2 complex (Janscak *et al*, 2001). The Res subunits couple ATP hydrolysis to movement of the helicase domain along the polynucleotide substrate (Bourniquel and Bickle, 2002; Sistla and Rao, 2004), and use a single endonuclease domain to make a single-strand DNA cleavage (Janscak *et al*, 2001). Comparison of the Mod subunits with DNA methyltransferases of known structure (Dryden *et al*, 2001; Sistla and Rao, 2004) shows that they comprise a standard S-adenosylmethionine binding and catalytic domain, together with a second domain responsible for sequence recognition (target recognition domain, TRD). We propose a structure for type III R/M enzymes similar to that of the type I R/M enzymes, which has the benefit of some plausible evolutionary pathway to its formation.

The Mod subunits read the recognition sequence from opposite directions, such that both strands can be checked simultaneously. The asymmetry of the target requires that the two Mod subunits be offset from each so that they do not read the same length of DNA at the same time. Offsets of six or seven bp are found for the bipartite DNA sequences recognised by type I R/M specificity subunits and also in the structure of *EcoR124I* methyltransferase (Obarska *et al*, 2006), so type III R/M enzymes may behave similarly. With the arrangement of the two Mod subunits shown in Figure 6A, it is apparent that the Mod_2 core of a type III R/M enzyme resembles the methyltransferase core of a type I R/M enzyme. Comparison with DNaseI footprints (Mucke *et al*, 2001) supports this structure. On addition of ATP, the protection around the rightmost (i.e., leading) Res subunit is altered. This indicates a conformational change, resulting in the activation of this Res subunit. The Res endonuclease domains lie at opposite ends of the molecule, as they do not cooperate with each other to perform cleavage (Janscak *et al*, 2001). Instead, two endonuclease domains, one from the enzyme bound to one target site and a second from another enzyme bound at the properly-oriented second target site cooperate to make a double-strand cleavage. The spacing

of the two Mod subunits plus the endonuclease domain offers an attractive explanation of why the cleavage sites of type III R/M enzymes are so far from the unmethylated target site sequence.

Functional implications for head-to-head site determination

The crux of the model is how a second tetramer is brought into contact in the correct orientation for cleavage of DNA only between asymmetric recognition sequences oriented in a head-to-head manner. For *EcoP15I*, the recognition sequence for cleavage can be considered to be 5'-CAG CAG(*N*)_{*n*}CTGCTG-3', where the sites are separated by a large number (*n*) of any nucleotide, *N*. Purified type III Mod subunits from the *EcoPI* enzyme are very similar to those of *EcoP15I* and can assemble into dimers and tetramers (Hornby *et al*, 1987). The ability of a Mod_2 dimer to itself dimerise suggests that Res_2Mod_2 complexes of *EcoP15I* may be able to do the same to form a $(\text{Res}_2\text{Mod}_2)_2$ dimer and this is our interpretation of the observation of single, large protein complexes on two-site DNA substrates (Figure 6A). This dimerisation process is likely to be rapid. We further postulate that dimerisation occurs in a specific orientation with the recognition sequence bound Mod subunits from each complex in contact. This places the Res subunits in contact with each other. Dimerisation is also observed with the type I R/M enzyme, *EcoKI*, when it is bound to DNA containing two recognition sequences (Ellis *et al*, 1999).

In addition, we propose that ATP hydrolysis by the helicase domains of the Res subunits allows them to act as transient clamps to grasp and release DNA segments that contact the helicase domain by 3D DNA looping. Thus, each Res_2Mod_2 complex has three DNA-binding components; the Mod_2 core plus the two Res subunits. For linear DNA, this model produces up to two loops for a single enzyme bound to a single-site DNA molecule as long as the enzyme is sufficiently far from the free ends of the DNA, up to four loops on a two-site DNA in the absence of dimerisation, and up to five loops for an enzyme dimer bound to a two-site DNA molecule. These three binding sites on each Res_2Mod_2 complex give rise to the loops labelled 1, 2, 4 and 5 in Figure 6B. The size of the DNA loops is governed by a continuous probability distribution, so it is unlikely that looping events will bring the two Res subunits into active contact on the DNA contour as required for cleavage; they will be still be separated by 'loop 3' in Figure 6B. We further propose that the 'fine tuning' to achieve cleavage is accomplished by limited ATP-dependent translocation of loop 3 once the bulk of the intersite distance has been reduced through formation of the other loops 2 and 4 in Figure 6B. In other words, a reduced distance is being translocated by a helicase with a more typical efficiency of order one bp translocated per ATP hydrolysed, accounting for the anomalously low rates of ATP hydrolysis observed previously (Meisel *et al*, 1995; Sears *et al*, 2005).

If the two recognition sites are not in a head-to-head orientation, separated by a Lac repressor sequence to which a repressor protein is bound (Meisel *et al*, 1995; Raghavendra and Rao, 2004), or if the sites are on separate DNA molecules linked in a catenane, then no cleavage occurs (Peakman and Szczelkun, 2004). These observations have been used to rule out a model relying solely on 3D diffusive looping, because this process is unable to specify the orientation of the second

Figure 6 Structural model for DNA cleavage by *EcoP15I*. (A) The structure of the Mod₂Res₂ tetramer bound to the recognition sequence. The Res subunits are shown as red spheres, the Mod subunits as green rectangles (the light green portion represents the catalytic methylation domain and the darker green portion represents the TRD). The left most TRD is responsible for reading the top strand and the right most the lower strand. The yellow arrow represents the *EcoP15I*-recognition sequence and the purple line represents the DNA cleavage site 25 bp from the adenine in the recognition sequence on the top strand and 27 bp on the bottom strand. The DNase I footprints on each strand in the presence and absence of ATP are shown in blue (Mucke *et al*, 2001). On the right, the proposed dimer of *EcoP15I* bound to two copies of the recognition sequence is shown. The rear molecule is semitransparent to allow the DNA and target site to be seen. (B) Cleavage of a head-to-head two-site template via DNA looping and translocation accomplished by the *EcoP15I* tetramer. Each Res subunit has a DNA-binding site allowing two loops per tetramer labelled 1, 2, 4 and 5, and a loop (labelled 3) resulting from the dimerisation of the tetramers (we have not shown the dimerisation for clarity). By this mechanism, the intersite distance is reduced before the enzymes are brought together by translocation. The leading Res subunits from each tetramer (shown enlarged) then cooperate in double-strand DNA cleavage. DNA segments within the loop caused by 3D looping are shown in blue and those caused by translocation are shown in orange. (C) The influence of dimerisation of *EcoP15I* on the DNA loops formed by head-to-head-cleavage competent substrates and head-to-tail cleavage-defective substrates. The translocating Res subunits are shown enlarged. Translocation leading to contraction of loop 3 in the head-to-head complex pulls the loop taut between the two translocating Res subunits and we postulate that this is the trigger for cleavage. Translocation of loop 3 in the head-to-tail complex pulls loop 3 taut across the whole complex and we postulate that this does not trigger cleavage.

DNA contact when the loop contour is larger than the persistence length. However, these experiments do not rule out our model, as the dimerisation and the DNA translocation will be directional even though the diffusional looping cannot impose an orientation.

For clarity, we will ignore the motors leading to the formation of loops 1 and 5 in Figure 6B and consider only one motor in each enzyme. If loop 2 is at least several persistence lengths long then the helicase motor in the Res subunit can grab either the top strand or the bottom strand

with equal probability and commence translocation in a 5′–3′ direction. If it translocates on the top strand then loop 2 expands, if it translocates on the bottom strand then loop 2 contracts and the enzyme has to restart the whole process again. A similar process can occur for the enzyme bound at the second recognition site, but this time loop 4 will expand if the motor is translocating on the bottom strand in the 5′–3′ direction and contract if it is moving along the top strand. Considering both translocating motors then, any pair of recognition sites can have both motors on the top strand,

both on the bottom strand or one motor on each strand. Only the last situation leads to the expansion of loops 2 and 4, and the contraction of loop 3.

Whether or not cleavage occurs after contraction of loop 3 depends upon the orientation of the recognition sites shown explicitly in Figure 6C. Head-to-head sites, which contract loop 3, are postulated to result in a successful cleavage complex. A head-to-head template will result in translocation of loop 3 by both of the active Res subunits. As no structure of a type III R/M enzyme is known, we have to postulate that the cleavage is only possible when loop 3 has contracted and is pulled taut between the two active Res subunits. In contrast, on a head-to-tail template, loop 3 is only being translocated by one of the Res subunits, the other Res subunit is not translocating on loop 3. Head-to-tail sites, which contract loop 3, clearly lead to a complex where the DNA is positioned differently, and presumably incorrectly, stretched across the protein complex from the left to the right hand side.

Intramolecular 3D looping events will be rare on plasmid and linear DNA as the probability of looping within the same DNA molecule will always be higher, even in relatively concentrated solutions. This will not be the case on catenated DNA with one site per catanane, as the two catananes are held proximal to each other. If the Res of one complex were to loop onto the opposite DNA catanane and associate with the other Res subunit, it is conceivable that cleavage could occur. However, in this case, the first complex remains bound to the second catanane by only the translocation domain of one Res subunit. The rest of the complex (presumably including the residues responsible for DNA cleavage) remains bound to the first catanane. It is difficult to see how such a scenario could lead to double-strand cleavage on either of the catananes. This is in accord with experimental evidence showing *EcoP15I* is unable to cleave catenated DNA substrates (Peakman and Szczelkun, 2004). For a bound repressor protein, the situation is slightly less clear. The formation of a 3D loop by *EcoP15I* may be able to loop out the bound Lac repressor; hence, cleavage activity should persist when the enzyme forms a suitably large loop. In our experiments, we observe that cleavage activity persists for Lac-binding site-*EcoP15I* spacings of 1.75 to 2 kbp. Loops larger than this will not be infrequent, thus it seems likely that *EcoP15I* may be capable of looping out a bound Lac repressor. Alternatively, *EcoP15I* may be able to occasionally displace bound Lac repressor or simply wait for it to dissociate and translocate past the Lac-binding site.

The above discussion has only dealt with a subset of all looping scenarios. Within the framework of our model, it is unclear what happens when loop 2 is longer than the intersite separation, so the enzyme 'loops over' the second site, or when a Res subunit loops DNA on the opposite side of the complex. It is also possible to devise alternative dimerisation, looping and translocation mechanisms to distinguish between head-to-head and head-to-tail site orientations, but detailed structural information would be required to distinguish between these schemes. Despite these uncertainties, what is clear is that the model must include 3D looping to explain the experimental evidence, namely the size and number of loops and the anomalously low ATP-hydrolysis rates. Our model goes some way towards solving, but does not completely solve, the apparent impasse surrounding the mechanism of type III R/M enzymes.

Functional implications for *EcoP15I* and other restriction enzymes requiring two sites for activity

Many restriction enzymes require two copies of their DNA-recognition sequence for optimal activity (Mucke *et al*, 2003; Halford *et al*, 2004). The problem faced by such enzymes is to bring together two enzyme molecules as rapidly as possible to bring invading phage or plasmid DNA under control. The kinetic factors to be considered (Figure 7) include the rate of loop formation by random intersegment collisions within the DNA molecule, the rate of the protein conformational change required to recognise the second site after collision, random 1D sliding rates, ATP-hydrolysis-driven translocation rates and processivity. The rate of loop formation by random intersegment collisions has been shown by theory and simulation to be rapid (millisecond timescale) for loops longer than ~400 bp (Hyeon and Thirumalai, 2006). It is likely that protein conformational changes to grab hold of the DNA to form a loop will be on the ms to s timescale, whereas 1D random sliding will be very fast (Halford and Marko, 2004). Initiation of translocation is very slow (second time scale) for type I R/M enzymes (Seidel *et al*, 2004, 2005; McClelland *et al*, 2005; Stanley *et al*, 2006), but once started the translocation proceeds in a directional and processive manner at rates of 100–1000 bp per second (Studier and Bandyopadhyay, 1988; Davies *et al*, 1998).

For the type II restriction enzymes requiring two sites, the site-to-site orientation is not relevant, as their target sequences are palindromic. The type II restriction enzymes that require two sites do not have the benefit of ATPase-driven translocation, so must rely upon 3D looping and limited random diffusion along the DNA contour to bring the two sites together (Figure 7). After dimerisation of two site-bound enzymes, the cleavage activity of the enzyme is activated (Catto *et al*, 2006). Although this is cheap from an energetic point of view, the random nature of the search may be slow if the rapid looping process is followed by a slow conformational change to recognise the second site.

The type I and type III R/M enzymes recognise asymmetric target sequences and the type III require a defined site-to-site orientation for activity. The type I R/M enzymes achieve their activity by binding a single enzyme to each of two recognition sites and then performing a directional and highly processive DNA translocation to bring the two sites together, (Figure 7). This is a very costly process as approximately one ATP molecule is required per bp translocated and the sites can be separated by many thousands of bp (Eskin and Linn, 1972a,b; Endlich and Linn, 1985; Davies *et al*, 1998, 1999; Bianco and Hurley, 2005). Nevertheless, it does have the benefit of inevitably bringing the two sites into contact and triggering cleavage.

Our observation of 3D looping and translocation by type III R/M enzymes suggests an intermediate situation between type I and type II restriction enzymes. We propose that the type III R/M enzymes have found an optimal search strategy to bring two distant sites together in a defined orientation. Halford and Marko (2004) have shown that the most efficient method for a protein to locate a specific target on DNA is by 3D diffusion to bring the protein and the target sequence into close proximity, followed by random 1D sliding for distances of about 100 bp (Halford and Marko, 2004). This model should apply to each type III R/M enzyme as it finds its target sequence. Once bound, further diffusion of the DNA

Figure 7 Search strategies used by restriction enzymes requiring two target sites for cleavage. Type I R/M enzymes only use 1D ATPase-driven processive translocation to close the distance between sites and to ensure correct orientation of the DNA in the cleavage complex. One enzyme binds to each of the two sites. Type IIE and IIF enzymes bind to a single site and then rely upon 3D diffusional looping to bring a second segment of DNA into contact. Upon loop formation, a limited amount of 1D diffusional sliding takes place. If a second target sequence is located during this sliding, before break down of the loop, then cleavage occurs. Type III R/M enzymes combine elements of both type I and type IIE/IIF enzymes. Two copies of the enzyme bind to target sequences on the DNA. 3D diffusional looping occurs bringing the two enzymes closer together. ATPase-driven translocation may start before the loops break down and this brings the two enzymes closer together until collision and cleavage occur.

contour not only allows rapid protein dimerisation, but also brings other regions of the DNA molecule into frequent contact with the DNA, thus forming DNA loops. We propose that when such a loop is formed, a region of DNA is scanned by limited *and* directional 1D translocation driven by ATP hydrolysis. During this process, if the loop linking two type III R/M enzymes oriented in the opposite direction on the DNA is correctly translocated, then cleavage will be triggered by juxtaposition of a Res subunit from each enzyme. In this manner, ATP hydrolysis is minimised whereas large separations between recognition sites can be rapidly traversed and the relative orientation of the sites determined.

Materials and methods

*Eco*P15I was purified as described previously (Raghavendra and Rao, 2004). Template SS (2249 bp) and HH (4994 bp) were generated by digesting plasmid pTYB1 (NEB, UK) with *Sfo*I and *Eco*RI, or *Pvu*II and *Bgl*II, respectively. The *Eco*P15I site at 3566 bp on plasmid pTYB1 was changed to 5'-CACCAG-3' (mutated base shown in bold) using the Quikchange site-directed mutagenesis kit (Stratagene, UK) with primers 5'-GCTGGTTTGCCCCACCAGGCCGA AAATCCTGTTGA-3' and 5'-TCAAACAGGATTTTCGCTGGTGGG CAAACCAGC-3', to yield pTYB1-+. The *Eco*P15I site at 7382 bp on plasmid pTYB1-+ was then mutated to 5'-CACCAG-3' using primers 5'-GGAAGCTGAGTTGGCTGGTCCACCGCTGAGCAAT-3' and 5'-ATTGCTCAGCGGTGGCACCAGCAACTCAGCTTCC-3' to yield pTYB1-+. The orientation of the *Eco*P15I site at 3566 bp on plasmid pTYB1 was inverted by mutation to 5'-CTGCTG-3' using primers 5'-GTCCACGCTGGTTTGCCCCGCTGGCGAAAATC-3' and 5'-ATTTTCGC CAGCAGGGGCAAACCAGCGTGGACC-3' to yield pTYB1 HT. Template +, template - and template HT were generated by digesting the appropriate plasmid with *Pvu*II and *Bgl*II. All fragments were then gel purified using the Qiaquick extraction kit (Qiagen, UK).

*Eco*P15I-DNA complexes were formed by incubating 35 ng of DNA with *Eco*P15I at a 1:1 recognition site:enzyme ratio in either

low-salt buffer (5 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 5 mM KCl) or high-salt buffer (20 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 50 mM KCl) supplemented with 80 μ M S-adenosyl-L-methionine (SAM) in a final volume of 20 μ L. ATP γ S (1 mM) or ATP (1 mM) (Sigma, Poole, UK) was added after 2.5 min and incubated for a further 2.5 min at room temperature. A 10 μ L portion of the reaction was then deposited on freshly cleaved muscovite mica (Agar Scientific, UK), incubated for 2.5 min, rinsed in milliQ water (Millipore, MA, USA) and dried in a stream of dry nitrogen.

Samples were imaged with a Nanoscope IIIa Multimode atomic force microscope (Veeco, Santa Barbara, CA, USA) operated in tapping mode in air with a scan range of 3 μ m. Olympus OMCL-AC160TS tips (Olympus, Tokyo, Japan) were used throughout. The position of the enzyme on the DNA was measured between the centre of the complex and the nearest end of the DNA strand, and was approximated as a series of straight lines using the Nanoscope software. The loop apex angle was measured as the angle between tangents extending from the centre of the enzyme and each of the DNA arms forming the loop.

Lac repressor protein was purified as described previously (Kumar *et al*, 1999). For AFM experiments, 35 ng of DNA was incubated in the presence or absence of *Eco*P15I at a 1:1 recognition site:enzyme ratio in Lac buffer (10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 10 mM KCl) supplemented with 80 μ M SAM and 8 ng Lac repressor, corresponding to a 5:1 Lac:DNA ratio in a final volume of 20 μ L. ATP (1 mM) was added after 2.5 min. Gel assays used 75 pmoles pTYB1 DNA and a 60- or 120-fold excess of repressor under identical buffer conditions. To abolish Lac repressor-DNA binding isopropyl-beta-D-thiogalactopyranoside (IPTG; Sigma-Aldrich) was added at a final concentration of 400 μ M. All reactions were treated with 20 μ g Proteinase K (NEB) and 0.1% SDS for 30 min at 37°C, and stopped with 10 μ M PMSF.

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