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Mechanistic insight into Type I restriction endonucleases

James Youell[1], Keith Firman[2]

[1]IBBS Biophysics Laboratories, School of Biological Sciences, University of Portsmouth, St. Michael's Building, White Swan Road, Portsmouth PO1 2DT, Hampshire, United Kingdom, [2]Now retired from School of Biological Sciences, University of Portsmouth, King Henry Building, King Henry I Street, Portsmouth PO1 2DY, Hampshire, United Kingdom

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1. ABSTRACT

Restriction and modification are two opposing activities that are used to protect bacteria from cellular invasion by DNA (e.g. bacteriophage infection). Restriction activity involves cleavage of the DNA; while modification activity is the mechanism used to "mark" host DNA and involves DNA methylation. The study of Type I restriction enzymes has often been seen as an esoteric exercise and this reflects some of their more unusual properties – non–stoichiometric (non–catalytic) cleavage of the DNA substrate, random cleavage of DNA, a massive ATPase activity, and the ability to both cleave DNA and methylate DNA. Yet these enzymes have been found in many bacteria and are very efficient as a means of protecting bacteria against bacteriophage infection, indicating they are successful enzymes. In this review, we summarise recent work on the mechanisms of action, describe switching of function and review their mechanism of action. We also discuss structural rearrangements and cellular localisation, which provide powerful mechanisms for controlling the enzyme activity. Finally, we speculate as to their involvement in recombination and discuss their relationship to helicase enzymes.

2. INTRODUCTION

As their name suggests, Type I Restriction–Modification (R–M) enzymes were the first discovered and provide bacteria with an adaptable and efficient system of protection against invasion by "foreign" DNA (bacteriophage and plasmids) by means of the two separate functions of restriction (1-3) – recognition and cleavage of "foreign" DNAand modification (4) – marking of host DNA using a specific methylation activity (3), which allows host DNA to be differentiated from "foreign" DNA (5). Type I R–M enzymes are capable of both functions and, as a consequence, must switch between these activities as appropriate.

These R–M enzymes recognise short (6-7 bp) DNA sequences (see below for details) that are comprised of two specific regions separated by a non-specific spacer (6) and the enzyme binds specifically at these recognition sequences (7), but DNA cleavage can occur many thousands of base pairs away from this binding site and appears to be a random process. In contrast, the modification activity involves a site-specific methylation at the N-6 group of adenine (8) and occurs within the recognition sequence (8).

2.1. Genetic organization and nomenclature

The genes that encode Type I restriction enzymes were named host specificity determinants (*hsd*) genes (9) and consist of three genes (10-11):

hsdR is absolutely required for restriction (DNA cleavage) activity and produces the HsdR subunit, which also binds the cofactors ATP and Mg^{2+}. Two HsdR subunits are required for restriction activity in the fully functional R–M enzyme (holoenzyme). This gene is expressed from a single promoter Pres (6).

hsdM is required for production of the HsdM subunit responsible for binding of the cofactor S–adenosyl methionine (AdoMet) – the methyl group donor – and is responsible for modification activity (12). Two HsdM subunits are present within the R–M enzyme allowing methylation of a single target adenine by each HsdM subunit.

hsdS is absolutely required for DNA binding and DNA specificity - determining the DNA recognition sequence of the enzyme(s) (10). A single HsdS subunit is present in the R–M enzyme allowing specific binding at the recognition sequence.

The *hsdM* and *hsdS* genes are expressed from a single promoter Pmod and they overlap by one base pair, producing a translational control mechanism that produces less HsdS protein than HsdM (13-14). All three genes are transcribed in the same direction, but the *hsdR* gene may be either upstream or downstream of *hsdM-hsdS*, depending upon the family group (see below and ref: 6). The *hsdM* and *hsdS* gene products are capable of producing an ATP–independent modification-only enzyme – a DNA methyltransferase (MTase) (15). However, all three gene products are required to produce a fully functional R–M enzyme (16-17). The stoichiometry of the enzyme is $HsdR_2$:$HsdM_2$:$HsdS_1$, while the independent MTase has a stoichiometry of $HsdM_2$:$HsdS_1$. However, as will be detailed below various sub–assembly stoichiometries have been detected, some with different functions.

A systematic nomenclature system was developed for all known R-M systems (18) and application of this system to Type I R–M enzymes (19) resulted in a few changes to previously published names (e.g. the Roman numeral I was placed after all Type I enzymes – EcoK became EcoKI, EcoR124 became EcoR124I). In addition, this systematic nomenclature suggests a mechanism for naming of the individual subunits of the Type R–M enzymes – e..g. HsdR(EcoKI) etc. In addition, the subunit name is often abbreviated to a single letter – HsdR becomes R, which leads to a description of the stoichiometry of the holoenzyme of $R_2M_2S_1$.

In summary, Type I R–M systems consist of three genes controlled by two promoters, Pmod is responsible for expression of the *hsdM* and *hsdS* genes, which encode the subunits that can assemble into an independent MTase. Production of HsdS is limited by a translation control mechanism resulting from an out of frame overlap of the two genes. Pres is responsible for the independent expression of the *hsdR* gene, producing the HsdR subunit, which is absolutely required for restriction activity and in turn requires the cofactors Mg^{2+} and ATP; however, Mg^{2+} and AdoMet are sufficient for DNA methylation by the independent MTase.

2.2. Families of type I restriction enzymes

Type I restriction enzymes have been divided into families based on their genetic organisation, antigenic cross reactivity and the ability of the subunits to complement for one another. The most notable feature of these families is that the chromosomally located genes (20) of three of the families Types IA, IB and ID are found in a region that has come to be known as "the immigration control" (21-22). This area is located early on the *Escherichia coli* chromosome (adjacent to the *serB* locus). To date, four distinct families have been identified:

Type IA – this family group is the best described and oldest family grouping in the literature, the archaetypical member being EcoKI (23), but also including EcoBI (24) and EcoDI (25). Many new members of this family group have been identified by protein sequence alignment studies (26), but their activity is yet to be ascertained.

Type IB – this group includes the EcoAI enzyme (27) and EcoEI (28), which have a similar chromosomal location the Type IA systems and an identical gene organisation, but do not complement the Type IA systems (as detailed within ref 28).

Type IC – this group has also been widely studied (primarily because of the ability to readily obtain high level protein production) and includes the archaetypical member EcoR124I and its closely related variant EcoR124II (formerly EcoR124/3) (29) as well as EcoDXXI (30), all of which are plasmid-bourne systems (31), and the chromosomal system EcoprrI (32).

The presence of a R–M system, on a conjugative plasmid, presents an additional requirement for the control of restriction versus modification as an exconjugate receiving the incoming plasmid must modify the chromosome BEFORE any restriction activity is observed (33-34). This mechanism of control is discussed in detail in a later section, however, it is important to mention something of this control at this early stage of this description – the EcoR124I enzyme was found, somewhat unexpectedly, to be a relatively unstable complex (35), which exists in solution as an equilibrium mixture of two complexes. The fully functional holoenzyme (with restriction and modification activities) consists of the expected $R_2M_2S_1$ assembly, but this readily dissociates into a relatively stable $R_1M_2S_1$ assembly and free HsdR with a Kd of 2×10^{-7} M (36).

Type ID – first identified as an hsd locus with the strain *Salmonella enterica* serovar blegdam (37), the founder member of this group is StySBLI. Further studies

identified EcoR9I (26) and KpnAI (38) as other members of this group.

In summary, Type I R–M systems have been characterised into four independent families based or different degrees of relatedness and gene organisation. They also recognise "split" DNA sequences, which consist of two specific regions on the DNA that are separated by a spacer region of non-specific nucleotides – e.g. EcoKI recognises AACnnnnnnGTGC (for a table of known recognition sequences see: http://www.typei-rm.info/genes.htm#Recognition_sequences).

The layout of the recognition sequences suggest two DNA binding domains within the specificity subunit HsdS and the adenine targets for methylation area are separated by one turn of the DNA (10 bp).

2.3. DNA methylation and cleavage

The single most distinguishing feature of Type I R-M enzymes is that they are capable of both restriction and modification activities (hence the name R–M enzyme) and the enzyme must be able to switch between these two opposing activities (39). However, the nature and longevity of this switch remains to be determined. It is clear that binding of the R–M enzyme to an unmethylated substrate enables an ATPase activity and subsequent restriction activity (24, 27, 40), but following DNA cleavage, methylation of the substrate DNA has been observed (41) suggesting the switch in activity is temporary, or reversible. Following DNA cleavage the ends of the DNA are released by the enzyme (24, 42), which allows access by recombination enzymes such as RecBCD that degrade the linear DNA produced (42), suggesting a useful role for these enzymes *in vivo* during recombination (43). This cleavage also converts covalently closed circular DNA (cccDNA) into linear DNA, which then also becomes an efficient substrate for recombination.

A variety of mechanisms have been determined as controls of restriction versus modification activities. In fact, in the simplest situation, it is the methylation status of the target DNA that governs the function of the R–M enzyme. The enzyme must read the methylation status of each of the adenines and communicate this methylation status – if both adenines are unmethylated (a situation indicative of invading DNA e.g. from a bacteriophage) then the enzyme switches to an endonuclease ENase (restriction) activity and will translocate and then cleave the DNA at a random site (44-45). However, if one of the adenines is methylated (a situation that will arise on the host chromosomal DNA following DNA replication) this information is transmitted to the HsdM subunit at the other adenine and methylation occurs (46-47). Finally, if both adenines are methylated the R–M enzyme will dissociate from the DNA (47). However, some family members have other control mechanisms, which will be detailed later.

Perhaps one of the most unusual properties of a Type I R–M enzyme is that the restriction activity has been described as stoichiometric (these "enzymes" are described as having no turnover and the enzymes appear as an oddity within the area of restriction enzymes) and yet Type I enzymes are perhaps the most prolific and adaptable systems known, which would belie this unusual property. Their ability to rapidly evolve new specificities (48-52) and to switch DNA specificity in a controlled manner (53) indicate evolution has produced a very adaptable and successful system, but a stoichiometric "enzyme" suggests a very inefficient system for protection of the bacteria, yet this is very far from the actual situation. This unusual observation has been resolved by Bianco *et al* (54), using a stable, purified EcoR124I holoenzyme (R–M enzyme – much of the previous work with EcoR124I has involved a reconstituted enzyme produced by adding purified HsdR to purified MTase), which has been shown to be truly catalytic. They determined that the DNA–bound enzyme, isolated after a cleavage event, was able to transfer to another DNA molecule, through a dimerisation event, to produce further cleavage – up to eight rounds of catalytic cleavage were readily measured (54). It is interesting to note that this work points to a dimerisation event (c.f. EcoKI, as described below), but it seems likely that this dimerisation may not be an absolute requirement for DNA cleavage for both enzymes (the reconstituted EcoR124I enzyme showed no dimerisation *in vitro* as measured by AFM techniques, KF unpublished observations).

As mentioned above, the HsdS subunit is responsible for DNA binding and a single copy of this subunit is present in both the R—M enzyme and the core DNA—binding MTase (57-59). The subunit was found to have a circular structure of repeating domains (60), which include two Target Recognition Domains (TRD) responsible for DNA recognition (Figure 1) of the bipartite recognition sequence (e.g. EcoR124I recognises $GAAn_6RTCG$), which are separated by two conserved domains (first recognised by Argos 1985 - see Ref No. 55 - and thought to be responsible for DNA binding, but later identified as responsible for protein-protein interactions) (61). TRD1 is responsible for binding the 5' portion of the recognition sequence, while TRD2 binds the 3' end (28, 62-68). This circular structure of repeated domains was shown to be adaptable in a way that allowed different positions of the N- and C-termini (69) and supports the idea of a highly flexible system for determining DNA binding. A conserved tyrosine has been identified as being involved in recognition of the adenine within the 5'-end of the recognition sequence based upon the availability for crosslinking with a modified DNA substrate (70).

This structure of HsdS provides an interesting means for the generation of new DNA specificities (49, 51, 71), which allows Type I R–M enzymes to rapidly evolve against mutations on a bacteriophage that remove binding sites. The basis for these changes is the swapping of the DNA sequences that encode the TRDs between related *hsdS* genes (50, 72), but unequal crossing-over can also generate new DNA specificity by altering the spacer region of the recognition sequence (48, 51), while truncation of HsdS can lead to dimerisation of the "half—subunit" to produce two identical TRDs that recognise the same DNA

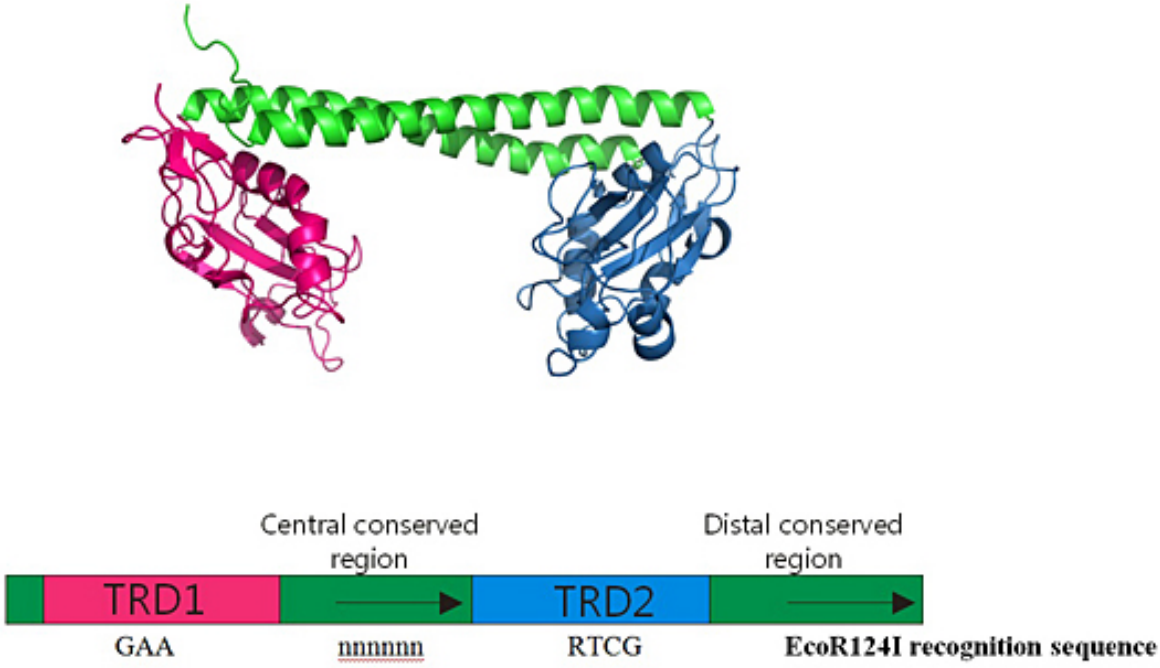

Figure 1. Repeated domains within the HsdS subunit. The HsdS structure from the pdb file at: ftp://genesilico.pl/iamb/models/MTases/M.EcoR124I/, shows TRD1 and TRD2 colour coded pink and blue respectively. The coiled-coil at the top (green) are the conserved regions. Below is a cartoon illustrating the simple structure of the HsdS subunit where the black arrows represent the Argos repeats (55) now known to be involved in protein-protein interactions (56) and in pink and blue the two Target Recognition Domains (TRD) that are responsible for DNA binding. Beneath the cartoon representation of HsdS is the recognition sequence for the Type IC R–M enzyme EcoR124, which shows that TRD1 is responsible for recognition of the 5'–end of the recognition sequence, while TRD2 recognises the 3'–end.

sequence, but on different strands (e.g. GAAn7TTC for a truncated version of EcoR124I) (52). Finally, the adaptability of these enzymes is wonderfully illustrated by the switching mechanism observed with the Type I R–M systems of *Mycoplasma pulmonis* (53), where inversion and recombination at a variety of sites within a group of *hsdS* genes allows up to 16 or more DNA specificities to be generated by a controlled switching mechanism (73).

It is clear that the core MTase is capable of independent binding to the recognition sequence and methylation of the target adenines within that recognition sequence (58-59), but the role (or even presence) of this independent MTase in vivo has been the subject of much speculation. The sites for methylation are the adenines within the recognition sequence – one on each DNA strand and, generally, these adenines are separated by 10 bp on the DNA, suggesting that they are on the same "face" of the DNA (56). However, this core MTase is also present in the RM enzyme and provides the holoenzyme with the same capability. Methylation substrate preferences vary slightly between families, with the Type IA enzymes showing a marked preference for hemi-methylated DNA (59), while Type IB and Type IC appear to be less selective and will methylate both unmethylated DNA (in the absence of ATP) and hemimethylated DNA (27, 58, 74).

The conserved region within HsdR, required for DNA cleavage, has been identified and termed motif X (37, 75) and a mutational analysis of this motif showed that restriction–deficient mutants lacked any nicking activity, but retained translocation and ATPase activity (76). This would suggest that each HsdR subunit carries a single nucleolytic site and could cleave a single strand of DNA, with double–stranded cleavage requiring the two HsdR subunits, which are found in the fully functional holoenzyme. One of the earliest studies of DNA cleavage by a Type I R–M enzyme was that of Horiuchi *et al* (77), who showed that cleavage by EcoBI occurred on one strand of the DNA, releasing acid–soluble oligonucleotides and produced a 75 nucleotide gap in the DNA. A second R–M enzyme was required to yield double–strand cleavage. This work was expanded upon recently with an investigation of DNA cleavage by EcoKI, EcoAI and EcoR124I (78). The release of staggered cuts with both 5' and 3' overhangs was confirmed for all enzymes (no blunt–ended fragments were detected) with EcoAI preferentially releasing short 3' overhangs, while EcoKI and EcoR124I released slightly longer 5' overhangs as their most frequent products. A motif X mutant of the EcoAI enzymes showed a nicking activity, which supports the idea that two HsdR subunits are required for cleavage of double–stranded DNA. However, the problem with this simple model is that the two HsdR subunits, present on a

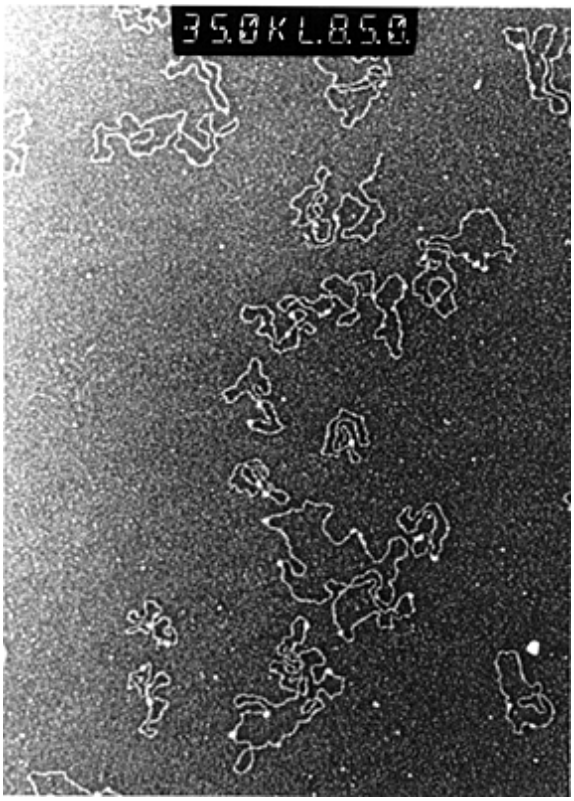

Figure 2. DNA cleavage follows DNA translocation. (A) The R–M complex is represented by the blue oval and the red squares at the top of the cartoon (the grey inset square with C represents the DNA cleavage site – motif X (37)). Below is a representation of DNA as a solid black line with a yellow rectangle that is the DNA recognition sequence for the R–M enzyme. (B) Binding of the MTase to the DNA recognition sequence initiates translocation as a result of ATP hydrolysis and where both HsdR subunits translocate (as illustrated) the DNA is drawn into the bound complex producing expanding loops of DNA. (C) A blockage on the DNA halts translocation and results in cleavage of the DNA.

single holoenzyme, translocate DNA from opposite ends of the enzyme and it seems unlikely that they can cooperate to produce a double–stranded cleavage event in one expanding loop of the DNA. Therefore, Jindrova *et al* (78) proposed a model for DNA cleavage that makes use of excess HsdR subunits that might available in solution, or present as part of an already assembled holoenzyme to ensure orientation of the two catalytic centres, and would enable staggered cuts of dsDNA, with random sized over hangs, as observed. In fact, this model has a lot in common with the catalytic model presented by Bianco *et al* (54) and the observed dimerisation of EcoKI (see later), which depends upon dimerisation of a translocating, DNA–bound enzyme and suggests that dimerisation may be an intrinsic aspect of the DNA cleavage mechanism (Figure 2), but there remain some problems associated with these models as dimerisation is not an absolute requirement for DNA cleavage.

In summary, Type I R–M enzymes are capable of both of the opposing functions of restriction (DNA cleavage) and modification (DNA methylation) and these two functions are controlled, or selected, by the methylation status of the target DNA – unmethylated DNA (usually a "foreign" DNA from outside the cell) is the subject of restriction activity. The R–M enzymes are also maintenance methylases and methylation of hemi–methylated targets follows DNA replication *in vivo*. DNA binding occurs through the HsdS subunit, which has a circular structure, two DNA binding domains and repeated conserved domains, which provide a highly adaptable capability for varying DNA specificity. DNA cleavage occurs at random location on the DNA, releasing linear DNA fragments with available, overhanging DNA–ends that are available for recombination. Catalytic activity has been observed through an unusual dimerisation process, which may also be the mechanism by which double-stranded cleavage occurs, with one HsdR subunit cleaving a single strand of DNA.

3. DNA TRANSLOCATION AND MOLECULAR MOTOR ACTIVITY

The random DNA cleavage activity is a complex process for Type I R–M enzymes and requires all three cofactors (ATP, AdoMet and Mg^{2+}). The process involves extensive ATP hydrolysis (79), which was shown to result in movement of the DNA substrate, through the bound enzyme (DNA translocation), producing expanding loops of DNA (80) (Figure 3).

A model describing a mechanism for DNA cleavage following translocation was first described by Studier and Bandyopadhyay (45) following synchronised initiation of ATP hydrolysis, which allowed them to map the site of cleavage at a point consistent with the position of collision of two translocating enzymes. Further studies have suggested that cleavage will occur following any process that halts DNA translocation including unusual DNA structures (81), or topological restraints on circular DNA (82-83). The outcome of this activity is that the point of DNA cleavage is random and the resultant fragments are of highly variable size (79). It is interesting that the presence of a Type I Restriction–Modification system increases the frequency of recombination in E. coli (84-85), which may provide some insight into why random cleavage is a useful strategy and supports the observation described above that the DNA ends of these random sized fragments are accessible to recombination enzymes (86-87).

3.1. Studies of DNA translocation using the Type IA R–M enzymes

EcoKI is the most widely studied of all Type I enzymes, and initial studies into the mechanism of DNA translocation involved a mutational analysis of regions of the *hsdR* gene that are conserved motifs associated with ATPases and helicases (see later) known as DEAD box motifs (88). This study allowed actual confirmation that the previously identified DEAD–box motifs (89) present in HsdR, were indeed required for restriction activity, the

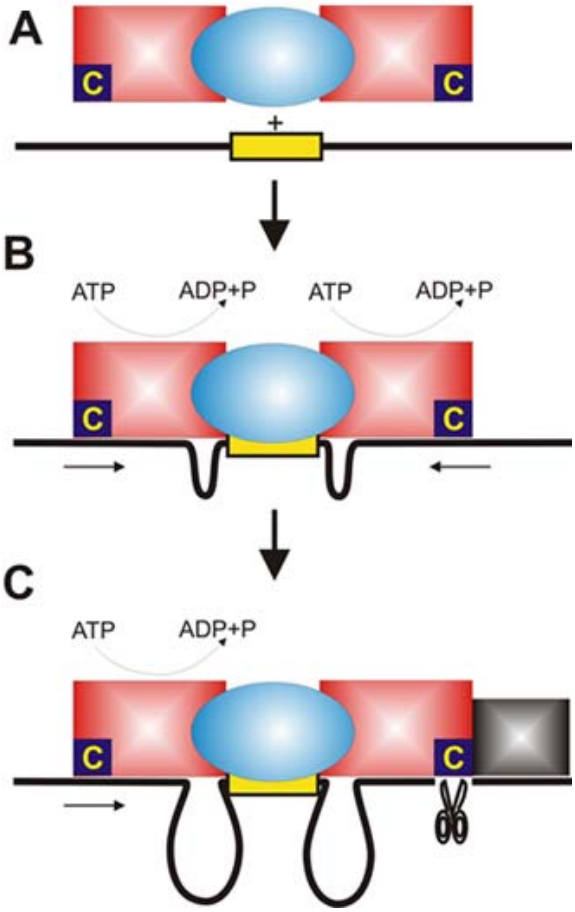

Figure 3. Expanding loops of DNA produced by translocation. Electron micrographs showing cccDNA following incubation with the Type IC R–M enzyme EcoR124I. Expanding loops, indicative of translocation, are highlighted by the red arrows.

mutants showed reduced ATPase activity, but were still able to nick the DNA (90). That this ATP hydrolysis is linked to translocation was confirmed by Davies *et al* (76) who showed that the restriction–deficient mutations within the DEAD–box motifs did indeed prevent translocation and that the observed nicking activity was uncoupled from translocation.

Attempts to analyse DNA translocation using single molecule techniques have provided only limited results and attempts to determine the characteristics of DNA translocation using a Magnetic Tweezer setup (91) were unsuccessful (David Dryden and David Bensimon, personal communication). However, imaging techniques involving use of the Atomic Force Microscope (AFM) were more fruitful (92-93). These studies analysed translocation by EcoKI, but these observations uncovered a surprising result – translocation–independent dimerisation of the enzyme (Figure 4) and DNA–loop formation. These dimers were particularly prevalent on linear DNA with two or more EcoKI binding sites (94) and led to a model of action for the enzyme. In this model, the binding of a second EcoKI molecule to a DNA–bound EcoKI is thought to greatly increase the efficiency of the search by the second enzyme for a specific recognition site. This process of dimerisation and searching for a binding site precedes the process of translocation, which does not occur until two DNA-bound enzymes are present as dimers and is thought to be the process described by Yuan *et al* (47) as an AdoMet–dependent switch to specific DNA binding. However, this dimerisation is not an absolute requirement for translocation and cannot provide the explanation for why measurement of translocation, within a Magnetic Tweezer setup, was unsuccessful. Neaves *et al* (94) suggest that this process of dimerisation, which would be an unlikely event *in vivo* involving the host chromosome, may provide a level of control that might prevent cleavage of the host chromosome (see later section on control of restriction). However, it is interesting to consider these observations in the light of the recent work by Bianco *et al* (54), where dimerisation of EcoR124I has been shown to enable catalytic activity in the DNA cleavage activity and the work of Jindrova *et al* (78) where such assemblies were thought to enable double stranded cleavage. Is this activity also true for EcoKI and does dimerisation provide a mechanism for function *in vivo*, which both allows a more efficient cleavage activity, but also enables a further level of control against cleavage of the host chromosome?

3.2. Studies of DNA translocation with the Type IC R–M enzyme EcoR124I

Perhaps the second most studied of the Type I R̄M enzymes is that from the Type IC family – EcoR124I, or its close relative EcoR124II (29). As described above and by Janscak et al (36), the EcoR124I R̄M enzyme is unusual in that the R2̄complex readily dissociates into an R1̄complex and free HsdR. The R1̄complex is observed at enzyme concentrations below 200 nM and at 20 nM enzyme concentration there is little or no observable R2̄complex. (36). This situation compounds studies with this enzyme and was discussed by Bianco and Hurley (42) in their studies of ATPase activity, with this enzyme. They described studies with a purified "native" enzyme, which appears to be very stable and not subject to this dissociation (Ref: 54 and personal communication) rather than the reconstituted holoenzyme produced by mixing MTase and HsdR. Bianco and Hurley (42) determined that ATP hydrolysis and DNA translocation were coupled and that 3 ATP molecules were hydrolysed per basepair of DNA translocated, but this work is confused by a low level of functional enzyme activity (16%-26%). The suggestion of DNA slippage was used to explain this relatively inefficient coupling, but a more likely explanation was differences in experimental conditions between ATPase measurements and those used to determine translocation rates as detailed below (Piero Bianco, personal communication). Interestingly, the ATPase activity of the enzyme was found to fall following cleavage (42), which may reflect methylation activity of the enzyme. In addition, the presence of RecBCD also lowered ATPase activity, due to degradation of linear DNA produced by restriction enzyme cleavage (54), which supports the observation, described

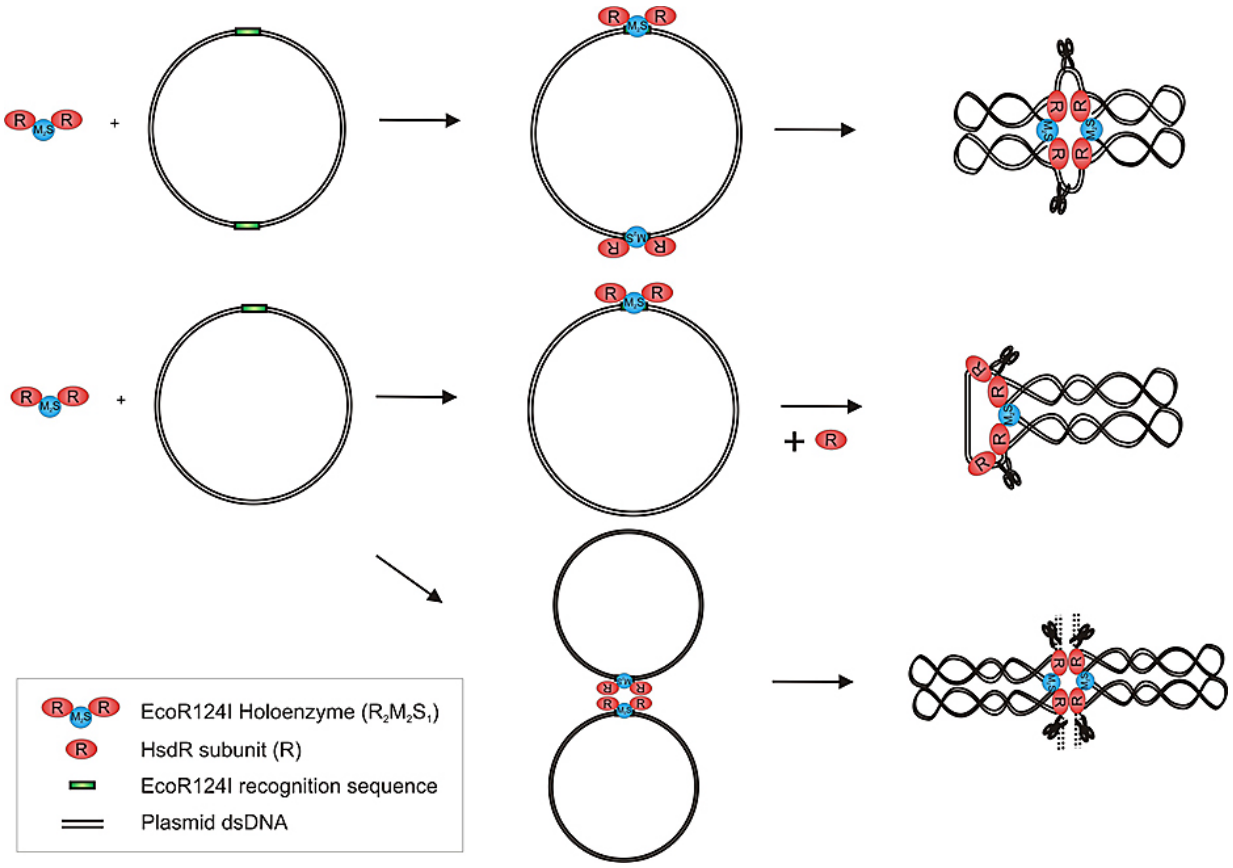

Figure 4. Dimerisation of Type I Restriction-Modification Enzymes. Top: Cleavage of a two-site plasmid substrate by interaction of the holoenzyme with another on the same substrate. The physical blockage of translocation by another holoenzyme (or other blockage on the DNA) results in double stranded nicking of the dsDNA substrate, as described by Studier and Bandyopadhyay (45) Middle: Cleavage of a single-site plasmid substrate by interaction of the holoenzyme with free HsdR molecules in solution results in double stranded nicking of the plasmid dsDNA as described by Jindrova *et al* (78). Bottom: Dimerisation of the holoenzyme results in translocation and double stranded nicking of the plasmid dsDNA as described by Bianco *et al* (54). The proposed mechanism has much in common with that proposed by Jindrova *et al.* (78) and observations of the translocation-independent dimerisation of EcoKI (92-93). All three mechanisms rely upon the translocation of the enzyme until a blockage of some kind is encountered whether another translocating enzyme, a physical blockage or a topological constraint in the DNA. However the nature of the initiation of translocation differs between the three mechanisms, with that proposed by Bianco *et al.* (54) requiring dimerisation of the holoenzyme before translocation and dsDNA nicking can occur.

earlier, that Type I R–M enzymes may have a role within recombination (87), but also indicates that, *in vivo*, the massive ATPase activity of these enzymes observed *in vitro* (40) may be regulated by this DNA degradation by the RecBCD enzyme to prevent cell death (54).

Therefore, Type I restriction-modification enzymes are powerful molecular motors, which use ATP hydrolysis to drive their translocation activity. The first detailed analysis of this motor activity was provided by a triplehelix displacement assay (95) in which a specific DNA sequence, to which a radiolabelled oligonucleotide could bind as a triple helix, was positioned at varying distances from the DNA recognition sequence of the Type IC restriction enzyme EcoR124I. Translocation by the enzyme was shown to displace the relatively stable triplehelix forming oligonucleotide and the time taken for displacement was measured following initiation of translocation using addition of ATP. This bulk assay showed that both the R_2–complex and the R_1–complex were both capable of translocation and the rate of movement of the DNA, by a single HsdR subunit, was 400 bp s^{-1}. Therefore, the two HsdR subunits are independent molecular motors, which are able to translocate DNA in opposing directions in an independent manner.

This analysis was improved by single molecule analysis of the process using a Magnetic Tweezer system (91). The data obtained from such experiments, using EcoR124I, largely confirmed the previous bulk studies (96), but showed that the actual rate of translocation was, in fact, slightly higher at 550 bp s^{-1} and that the difference between the observed rate from the bulk measurements and that obtained by the single molecule experiments was due

to periods of inactivity when no translocation occurred. The translocation was highly processive and initiated rapidly from stationary, but stopped equally suddenly with release of the bead (96). Again the observation from the bulk experiment that the two HsdR subunits were capable of independent translocation was confirmed and it was found that the R_2–complex was more processive. Further analysis of the resetting process, where the magnetic bead was found to be released from the translocating enzyme, showed that this resetting was a result of the dynamic nature of the EcoR124I enzyme and that it was in fact produced by dissociation of the HsdR subunit (97). Interestingly, a similar situation may have been observed with the Type IA enzyme EcoBI (44), where electron microscopy revealed unidirectional translocation and a reduced–sized R–M enzyme, which appeared to de deficient in at least one subunit. However, dissociation of EcoKI does not appear to be a frequent event.

Translocation leads to changes in the twist of the DNA (82, 96, 98) indicating that the enzyme follows the helical thread of the DNA. This reflects the organisation of the motor where the core DNA–binding component (the MTase) remains bound at the recognition sequence while the HsdR subunit, which remains in contact with MTase, pulls the DNA through this bound complex. Therefore, for each helical turn of DNA translocated there will be one negative supercoil created in the translocated DNA loop that exudes through the enzyme and one positive supercoil created ahead of the motor (96). This situation was confirmed by Magnetic Tweezer experiments using covalently closed DNA, which also allowed a twist step size (99) of 11 bp to be determined (96) – a figure that confirms that the motor follows the helical thread of the DNA – and suggests a small step-size for translocation 1-2 bp (100).

There is an interesting question that arises out of these observations and that is how does translocation initiate? The persistence length of DNA is approximately 50 nm (101), but the footprint of a Type I Restriction–Modification enzyme is much less that 50 nm (102-104). Therefore, how does HsdR bind an additional region of the adjacent DNA and initiate translocation? This question was answered by further single–molecule analysis involving a Scanning Probe Microscope (SPM) a technique used to visualise single molecules using nanoscale topography measurements (105-106). The SPM was used to visualise pre-translocation complexes, which were stalled using the non–hydrolysable ATP analogue ATP–γ–S (107) and two important observations were made – the first was a 20 nm shortening in the length of the DNA molecule, but the more important second observation was a small "bulge" of ssDNA, which was determined to be produced by a localised melting of the DNA in this initiation complex.

In summary, a Type I Restriction–Modification enzyme consists of a DNA–binding methylase complex (M_2S_1), which will bind tightly to the specific recognition sequence of the enzyme and two HsdR subunits that bind to the MTase. The HsdR subunits are independent, ATP–driven molecular motors, which can grasp DNA adjacent to the specifically bound methylase complex, by means of a local DNA melting event, which allows initiation of DNA translocation. Translocation moves the adjacent DNA, through the DNA–bound complex at 550 bp s^{-1}, until something blocks that process (local DNA topology, or another translocating enzyme). If the translocation process is halted in this way DNA cleavage can occur and will produce random–sized DNA fragments. The cleavage event has been described as stoichiometric, non–catalytic event, but this has recently been disproven and dimerisation of the enzyme has been shown to occur, to be translocation-dependent and to result in a catalytic behaviour during DNA cleavage. This dimerisation has been postulated for several situations and may be an intrinsic part of the DNA translocation/cleavage mechanism, but was not observed for EcoR124I using single molecule analysis (SPM and Magnetic Tweezer analysis).

4. DNA TRANSLOCATION AS A HELICASE-LIKE ACTIVITY

It is clear from the information described above that the HsdR subunit is a "docked" molecular motor similar to the RuvB helicase (that relies on the RuvA protein for Holliday junction recognition and docking) (108). However, there is no observed motor activity associated with the purified HsdR subunit; although the HsdR subunit of EcoR124I has been shown to bind curved DNA (109). Despite this, the presence of "DEAD–box" motifs (Figure 5) within the protein sequence of HsdR (89-90, 110) has led to the classification of HsdR as a member of superfamily (SF) 2 DNA helicases (111). To some extent this definition of HsdR as a helicase is correct as suggested by the previously described triple–helix displacement activity. Observations that the EcoR124I R–M enzyme can translocate past a interstrand cross-link in the DNA substrate suggests that the translocation mechanism is not typical of a helicase and does NOT involve the normal unwinding of the dsDNA substrate (100). By definition, an active helicase (one that utilises ATP hydrolysis for activity) is a translocase. Despite the fact that EcoR124I does not appear to unwind dsDNA it still adheres to the existing characterisation parameters defined by Singleton *et al.* (112) as an active, highly processive translocase. The fact that EcoR124I has been extensively studied as an archetypal dsDNA translocase has led researchers to believe that it can provide insight into the mechanism of other related dsDNA translocases, such as the chromatin remodelling factors (100). In fact, EcoR124I contacts primarily the 3'–5' strand of the DNA, moving along this strand while maintaining only minor contacts with the 5'–3' strand for stability purposes (100) and this work suggests that the enzyme is able to translocate along short regions of ssDNA at the same rate as dsDNA. As observed with other dsDNA helicases (113), the Type I R–M enzyme makes important contacts with both the backbone and the DNA bases of the 3'–5' strand and consequently use the whole of the dsDNA structure for motor activity (100).

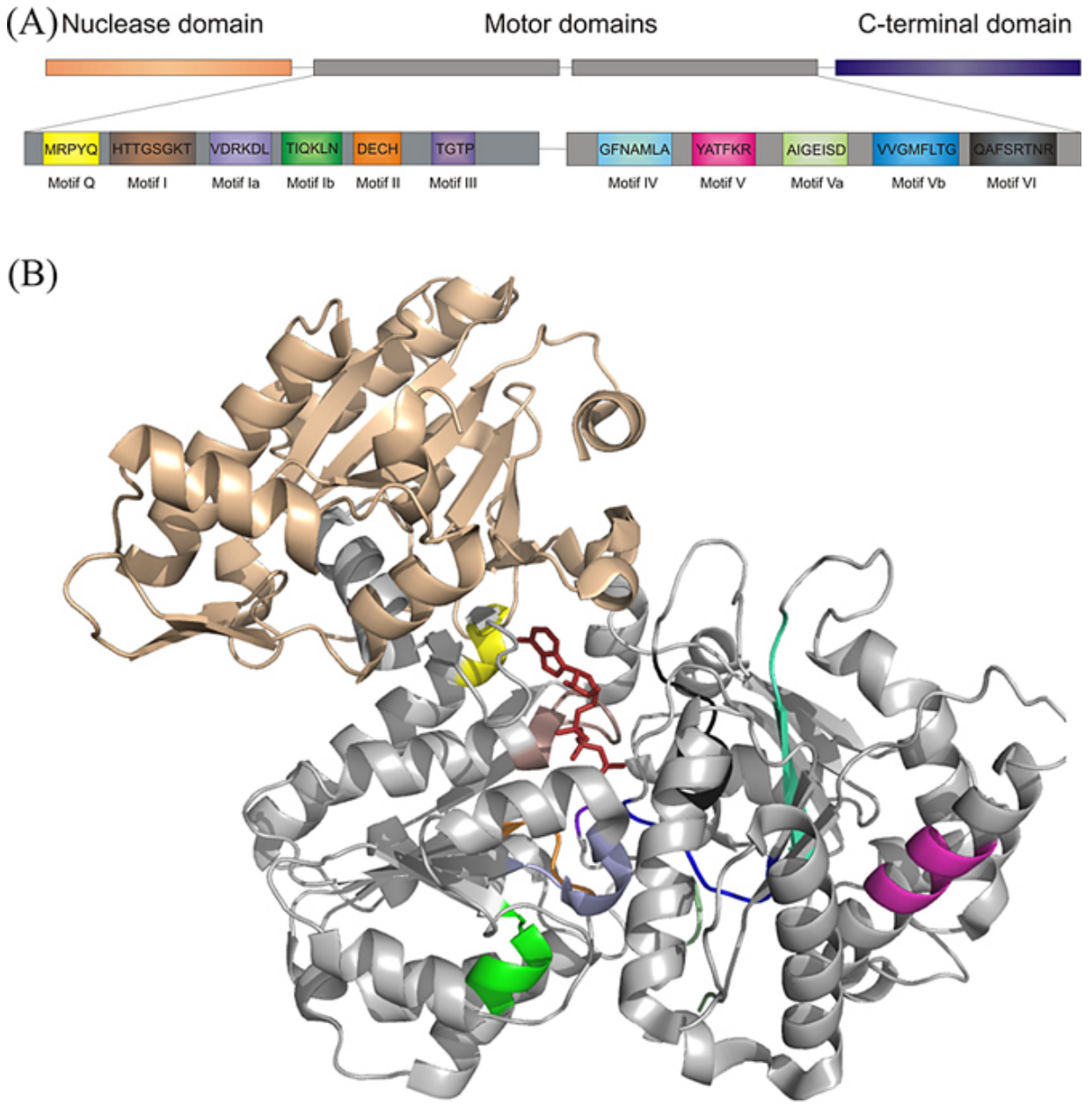

Figure 5. Location of helicase and nuclease motifs within the HsdR subunit. (A) Cartoon of the domain organisation of the EcoR124I HsdR subunit detailing the position of the RecB-like family nuclease domain, the two RecA-like "helicase" domains (and relevant motifs) and the C-terminal a-helical domain thought to be involved in interactions with the MTase domain. (B) Crystal structure of the HsdR subunit as determined by Lapkouski *et al.* (2008) (PDB at: http://www.pdb.org/pdb/explore/explore.do?structureId=2W00). The N-terminal nuclease domain and motor domain (RecA-I and RecA-II) are shown with helicase motifs annotated in a similar fashion to the cartoon.

However, inchwormlike models for DNA translocation (114), which involve a leading protein domain reaching along the DNA are incompatible with the observation that Type I RM enzymes can translocate over gaps as large as 300 nt (100). Although, it is possible that looping of the ssDNA region would allow such a model to operate, but this situation has been difficult to determine in a Magnetic Tweezer setup because of the effect of applied forces on the DNA substrate.

In summary, The HsdR subunit of a Type I R–M enzyme contains DEAD–box motifs associated with DNA and RNA helicases. Mutations within these DEAD–box motifs have clearly demonstrated their requirement for DNA translocation and the enzymes have been described as

members of SF2 helicases. Although the enzyme does not unwind dsDNA in the manner of a classic helicase, it behaves much like a "docked" helicase that is able to displace DNA strands and translocates in a 3'–5' direction following the 3'–5' DNA strand in a highly processive manner.

5. MECHANISMS FOR CONTROLLING RESTRICTION ACTIVITY

A Type I restriction–modification enzyme, as the name suggests, is capable of the opposing restriction and modification activities and must switch between these activities. As previously detailed, the primary mechanism for controlling restriction activity over methylation is determined by the methylation status of the target DNA. The modification activity acts to provide a maintenance methylation activity (115) of the host chromosome, methylating hemimethylated host DNA following DNA replication, which ensures that the host chromosome is always fully methylated. Unmethylated DNA usually comes from invading bacteriophage or plasmids and is recognised as "foreign" and subject to cleavage – the R–M enzyme switching to a restriction endonuclease upon detecting the unmethylated substrate DNA (47, 116).

However, there are other situations where the control of restriction versus modification activity becomes critical. Perhaps the most obvious is when a R–M system is first introduced into a new host cell, where modification activity must (and does) precede restriction activity (33-34). This was first observed using artificial systems that allowed conjugal transfer of the EcoKI system and the control was proposed to involve an unknown control gene, but this requirement for control is much more obvious with the plasmid encoded Type IC R–M systems (39). There are other situations in which modification of the host chromosome may not occur and one example has been called Restriction Alleviation (RA) and is observed where, following replication, the hemimethylated DNA is damaged in such a way that it is now unmethylated at the target site for a restriction enzyme, which should make it a target for the restriction activity of the R–M enzyme. However, this does not happen, the cell survives, and this RA event controls restriction activity *in vivo*.

5.1. Subunit assembly and control of restriction and modification

The Type IC R–M systems EcoR124I and EcoDXXI are resident on conjugative plasmids (30, 117) and as such present a unique problem for control of restriction and modification in that, as part of their natural situation, they must control restriction activity versus modification following conjugative transfer (EcoKI and other enzymes studied following conjugal transfer are not normally subject to such transfer on a frequent and natural basis).

This control was studied with the well described EcoR124I R–M system (39) following the observation that this R–M enzyme has an unusual subunit assembly pathway with a weak final R_2–complex (36). It is clear that EcoR124I can ensure modification of the host chromosome by means of the modification–proficient $R_1M_2S_1$ complex, which will assemble first at low concentrations of HsdR. The importance of this type of control, through subunit assembly, was also illustrated from the observation, with the closely related Type IC system EcoprrI (32), that an antirestriction activity produced by the presence of a small polypeptide (Stp) encoded by bacteriophage T4 (118) effected its anti-restriction activity by destabilising the R_2–complex (119) and releasing HsdR. The R_1–complex of EcoR124I was shown to be restriction-deficient (36), but modification–proficient, providing a very controlled mechanism for preventing restriction activity following phage infection by T4.

5.2. Restriction alleviation and Type I restriction–modification systems

Analysis of the timing of restriction activity versus modification activity following conjugal transfer of the EcoKI R–M system showed that restriction activity was delayed by approximately 15 generations following modification activity (33), which led to a proposal of a control gene (34), but no such gene was identified until an analysis by Makovets *et al* (120). However, the result of this analysis yielded a surprising result, which was that control was produced by the ClpXP protease, an essential component of an *E. coli* molecular chaperone system (121-123). They proposed that this protease may compete with the MTase for interactions with the restriction subunit HsdR.

This work was expanded upon by analysing an artificial mechanism for Restriction Alleviation in which DNA damage was induced at the recognition sequence for EcoKI using 2–aminopurine (124). This identified HsdR as a target for ClpXP proteolysis following induction of the artificial Restriction Alleviation situation from a variety of methods including 2–aminopurine. Interestingly, the loss of HsdR, due to ClpXP activity, only occurred when the HsdR was capable of assembly into a functional endonuclease – in the absence of HsdS and HsdM no degradation was observed. In fact, this work showed that only HsdR, present in a form where a commitment to restriction activity had occurred (reminiscent of the observation of dimerisation) is subject to this proteolysis (124), which supports the idea that a switch in function by the R–M enzyme results in a significant structural alteration to HsdR, which is recognised by ClpXP. However, the situation is more complex than this model suggests as Makovets *et al* also showed that invading bacteriophage DNA was still subject to some restriction activity, which suggests that localisation of this protease activity make be important (see below) – bacteriophage DNA and host DNA are located differently within the cell (124).

As discussed earlier, Type I R–M enzymes increase the frequency of recombination (84), which suggests they have a role within the cell beyond simple protection against invading bacteriophage. This concept

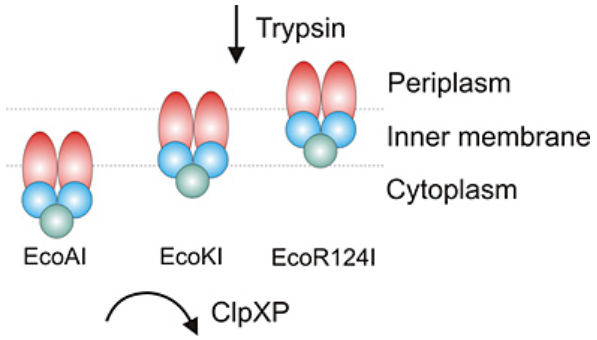

Figure 6. Cellular Organisation of the Type I Restriction–Modification Enzymes. The three R–M enzymes EcoAI, EcoKI and EcoR124I are members of the Type I families B, A and C respectively. The degree of periplasmic exposure of the respective HsdR subunits (open ovals) was determined by immunoblotting techniques following exposure to trypsin digestion. Holubova *et al* (123) suggest that this membrane localisation affords different levels of protection against the ClpXP protease, which has been found to be associated with Restriction Alleviation.

was investigated further in a *clpX* mutant strain where double–strand DNA breakage was initiated using 2–aminopurine treatment (RA) and the ability of RecG and RuvABC recombination systems to repair the DNA was determined (43). The two enzymes show differences in the mechanism for dealing with these DNA breaks and this has led to a new model for resolution of DNA breaks. These observation support the concept that Type I R–M enzymes provide DNA substrates that can be utilised by the homologous recombination systems of the host bacteria.

In summary, Restriction and Modification activities of a Type I R–M enzyme are opposing activities, one of which (restriction) could be detrimental to the cell and the other involves a maintenance methylation activity (modification). Therefore, fundamental to the existence of these multifunctional enzymes are mechanisms for switching between these two activities and controlling restriction activity. Three main mechanism have been observed for this – (i) a switch between modification and restriction activity triggered by the methylation status of the target DNA, (ii) control of restriction activity through subunit assembly of the fully functional R_2–complex, (iii) control of restriction activity of a translocating R_2–complex through the ClpXP protease, which acts to degrade the HsdR subunit present in a translocating R_2–complex.

6. CELLULAR LOCALISATION AND SUBUNIT ASSEMBLY

It is clear from the information above the control of restriction and modification activities is a key aspect of their mechanism of action, but that their dual role also suggest that these alternate functions may be in someway targeted toward their respective targets (incoming phage, or plasmid DNA, for restriction activity and the host chromosome for modification activity). This concept led to a detailed study of cellular localisation of Type I R–M enzymes based on separation of the periplasmic fraction from the cytoplasmic fraction and identification of the accessibility of the proteins within the membrane fractions by limited proteolysis and Western Blotting (125-126).

The initial study with EcoKI showed that HsdR and HsdM were found as soluble cytoplasmic proteins and were only found to associated with the cytoplasmic membrane when associated with the insoluble HsdS protein, while MTase and ENase were found to associated with the cytoplasmic membrane (126), but through an association with DNA (identified by means of benzonase treatment). Limited proteolysis of the spheroplasts showed that the HsdR subunit was exposed in the membrane–associated ENase, while HsdM and HsdS were both unexposed in both MTase and ENase (126). Further studies were subsequently carried out with representatives of the A, B and C families of Type I R–M enzymes and the only differences observed between the three families was the degree of periplasmic exposure of the HsdR subunit (Figure 6), as assayed by limited proteolysis (125). The most highly exposed HsdR subunit was that of the Type IC family – EcoR124I, followed by the slightly less exposed HsdR of the Type IA R__M enzyme EcoKI, but in stark contrast the Type IB enzyme EcoAI was not exposed to the periplasm to any extent and was refractory to benzonase treatment (125).

It is clear from these data that both MTase and ENase enzymes are associated with the cytoplasmic membrane as well as appearing free as soluble cytoplasmic proteins. This suggests that two versions of the ENase may exist within the cell. It is interesting to note that Holubova *et al* (125) showed that the membrane associated fraction of the available EcoKI was resistant to ClpXP digestion (Figure 6) and they propose that this fraction of EcoKI is targeted against invading DNA. The Type IC enzyme is not subject to ClpXP digestion, but control of restriction for this enzyme (as detailed above) is through subunit assembly (36). It is possible that HsdR-directed association of the ENase, with the cytoplasmic membrane, is a mechanism of localising the active ENase away from intermediate subassemblies (e.g. R1complex) that may be responsible of modification activity. The combination of cellular localisation and subunit assembly provides a very precise mechanism for post-translational control of restriction versus modification activity.

ClpXP proteolysis of HsdR was not observed for the EcoR124I Type IC system, but an interesting observation made following purification of the HsdR subunit of EcoR124I (109) was that this purified subunit was smaller than that observed in the intact endonuclease (35), but that this smaller HsdR subunit was also present in the preparation of the holoenzyme. N-terminal sequence analysis suggested that the smaller HsdR subunit was processed at the C–terminus, but it was unclear whether this processing altered the function in any way. It is interesting to reflect that this smaller HsdR subunit, used in preparation of all of the reconstituted holoenzyme work is not present in the native, purified holoenzyme used by Bianco *et al* (personal communication) and that this deletion may account for the observed instability of the reconstituted enzyme. However, despite these concerns, restriction and translocation activities of the reconstituted holoenzyme appear the same as those observed with the native holoenzyme (35, 54). The only major difference appears to be dissociation of HsdR from the R_2–complex. Therefore, the question remains does the native enzyme dissociate *in vivo* and is this dissociation a mechanism for control of restriction activity as discussed by Firman *et al* (39). The R_1–complex, produced by dissociation of the weakly bound HsdR subunit (36, 104) shows a restriction–deficient, modification–proficient phenotype (39), which supports the idea that after transfer of the plasmid R124, into a new bacterial cell, that modification would occur before assembly of the active holoenzyme. However, EcoBI has also been described a mixture of different stoichiometric complexes when purified (16) and alternative, non-functional assemblies of EcoKI have been identified (127), but these systems do not seem to depend upon this possible means for controlling restriction. The question remains do these alternative assemblies have any role *in vivo*? One indication that dissociation of EcoR124I may occur *in vivo* is that the T4 antirestriction polypeptide Stp (118) was shown to effect this activity by disassembly of the R2–complex or holoenzyme (119).

In summary, the Type I R–M enzymes have all been found to be associated with the cytoplasmic membrane, but the degree of exposure within the periplasm varies between the different families. This degree of exposure also correlates with sensitivity to the ClpXP protease responsible for RA, therefore, it is proposed that this localisation of the enzyme is important for targeting restriction activity against incoming DNA.

7. PERSPECTIVE

Type I R–M enzymes are multifunction and multisubunit enzymes that provide ideal models for the study of protein-protein interactions, DNA binding and the activity of molecular motors. Work to date on the control of the two opposing activities of restriction versus modification has already identified a complex system based on post-translational control, which may provide an interesting model for other systems. However, there is much yet to be understood and further work on the role of supramolecular assemblies of these enzymes and their role *in vivo* is required. Assays that localise activities *in vivo* would greatly assist in our understanding of how these complex enzymes function. It is also important to investigate further post-translational modifications to the enzymes (e.g. processing of the C-terminus of HsdR(EcoR124I), or phosphorylation of subunits (128) as observed with EcoKI). Yet further *in vitro* analysis, such as FRET labelling, may also finally elucidate allosteric and conformational changes that allow switching in function and assembly of complex dimers that enable DNA translocation If coupled to single molecule studies of translocation then it should be possible to determine if the switch between restriction and modification activities is permanent, what governs the switching and how frequently does the enzyme switch from translocation to methylation of target DNA. In addition, the involvement, or potential involvement, of these enzymes in recombination still remains to be fully understood and the recent analysis of how recombination systems deal with DNA damage following induction of Restriction Alleviation suggests more work is required in this area.

Recent structural studies of HsdS subunits from Type I R–M enzymes (129-130) led to the production of a structural model for the MTase of EcoR124I (56) and the recent publication of a crystal structure for HsdR (131) suggests a full ENase structure could be produced by modelling techniques as well as by ongoing studies using cyroEM. In fact, these systems would provide an ideal model for developing predictive algorithms for protein-protein interactions, which could be confirmed by other means. There is much scope for biochemical identification of protein-protein interactions, cross–linking of subunits and the final production of an accurate model of the quaternary structure of both the endonuclease and dimers thereof. The importance of dimerisation *in vivo* needs to be clarified and the purpose of this super-structure within the overall reaction mechanism needs to be properly resolved, is it an intrinsic part of catalytic cleavage, how do super-structures ensure double-stranded cleavage and what role might this dimer play *in vivo*.

However, it is undoubtedly the motor activity of Type I R–M systems which is the most exciting area for future work. The single molecule analysis using the Magnetic Tweezer system has shown the potential of the motor as a nanoactuator, pulling a magnetic bead (132). Work is already underway to develop an electronic version of the Magnetic Tweezer system, which may provide a novel biosensor. The question is can Type I R–M enzymes be used in such a device? In many ways Type I R–M enzymes behave like chromatin remodelling enzymes (KF, unpublished observations) and they would provide an ideal model system with which to develop mechanisms for better understanding the process of histone movement in chromatin. But their potential for analysis of DNA–binding drugs may be the area of greatest interest – only time will tell.

8. OVERALL SUMMARY

For many years Type I R–M enzymes have been seen as an enigma and a peculiar type of enzyme of very little real value to scientific study. Yet these enzymes are the most common R–M enzyme in bacteria and show an

amazing ability to both change DNA specificity by rapid evolution and to controllably switch DNA specificity to the benefit of their host organism. Nature does not design peculiarities and the fact that these enzymes are now recognised as a class of helicases, that they have a role within recombination and that they are an important model for understanding novel mechanism for post−translational control all suggest that the further study of these enzymes will provide fruitful outcomes.

9. NOTE ADDED IN PROOF

A recent paper by Simons and Szczelkun (133) has added a lot of information regarding the dissociation of HsdR and recycling of the subunits of Type I R-M enzymes. Of particular importance to this review is their observation that HsdR of both EcoR124I and EcoKI can recycle from DNA with a free end and rebind another DNA-bound MTase to perform further DNA cleavage. In addition, they found that both holoenzyme and reconstituted EcoR124I behaved identically and no turnover, induced by RecBCD was observed. Finally they observed recycling of the MTase of both EcoKI and EcoAI, but, as indicated above, not EcoR124I. This subunit turnover is thought to play a key role in Restriction Alleviation.

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Send correspondence to: James Youell, IBBS Biophysics Laboratories, School of Biological Sciences, University of Portsmouth, St. Michael's Building, White Swan Road, Portsmouth PO1 2DT, Hampshire, United Kingdom, Tel: 442392843077, Fax: 4423982053, E-mail: jim.youell@port.ac.uk