An investigation of the structural requirements for ATP hydrolysis and DNA cleavage by the EcoKI Type I DNA restriction and modification enzyme

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ABSTRACT

Type I DNA restriction/modification systems are oligomeric enzymes capable of switching between a methyltransferase function on hemimethylated host DNA and an endonuclease function on un-methylated foreign DNA. They have long been believed to not turnover as endonucleases with the enzyme becoming inactive after cleavage. Cleavage is preceded and followed by extensive ATP hydrolysis and DNA translocation. A role for dissociation of subunits to allow their reuse has been proposed for the EcoR124I enzyme. The EcoKI enzyme is a stable assembly in the absence of DNA, so recycling was thought impossible. Here, we demonstrate that EcoKI becomes unstable on long unmethylated DNA; reuse of the methyltransferase subunits is possible so that restriction proceeds until the restriction subunits have been depleted. We observed that RecBCD exonuclease halts restriction and does not assist recycling. We examined the DNA structure required to initiate ATP hydrolysis by EcoKI and find that a 21-bp duplex with single-stranded extensions of 12 bases on either side of the target sequence is sufficient to support hydrolysis. Lastly, we discuss whether turnover is an evolutionary requirement for restriction, show that the ATP hydrolysis is not deleterious to the host cell and discuss how foreign DNA occasionally becomes fully methylated by these systems.

INTRODUCTION

Type I DNA restriction endonucleases were the first restriction enzymes to be purified and characterized (1–6) but it was soon found that although they recognized a specific target DNA sequence they made a double-strand cleavage elsewhere on the same DNA molecule (7), typically thousands of base pairs distant and often approximately half way between successive target sequences (8) although sometimes close to a target sequence (9). Effective cleavage required one or more target sites on circular DNA and at least two sites on linear DNA (10). If multiple sites were on the DNA, atomic force microscopy images indicated that the enzymes could dimerise prior to the cleavage reaction (11,12). The endonuclease activity requires *S*-adenosyl-L-methionine (SAM), Mg^{2+} and ATP. However, the enzyme appeared not to turnover in the cleavage reaction such that each enzyme molecule only introduced a single double-strand cleavage (13,14). This absence of turnover means that the Type I restriction enzymes are often considered to be 'honorary' enzymes and most experiments use at least a 1:1 ratio of enzyme:DNA target sites. Loops of DNA were observed as the enzyme apparently held on to its target site while seeking a cleavage site via pulling in DNA toward itself using the energy from ATP hydrolysis (15–18). After cleavage the data suggested that the EcoKI and EcoBI enzymes were smaller in size than before the translocation reaction and it was suggested that they had lost some subunits (15,16,19). It is notable that subunits were not lost when a short oligonucleotide duplex was used to study the DNA footprint in the presence of ATP for the EcoKI restriction enzyme (20). It was also observed that ATP hydrolysis continued long after cleavage was complete but would be brought to an abrupt halt if the loops were cleaved by another endonuclease (21) or the DNA was degraded with DNaseI or RecBCD (21). DNA cleavage leaves a wide range of 5′- or 3′-overhangs of variable lengths (23–25) and a role for such variable fragments in recombination was proposed (25).

Structurally, the enzymes comprise one DNA specificity, S, subunit for target recognition, two modification, M, subunits that assemble with S to form an M_2S_1 methyltransferase (MTase) core, and two restriction, R, subunits to complete the bifunctional MTase/restriction endonuclease and act as a DNA restriction/modification (RM) system (26). The MTase activity methylates hemi-methylated target sites on host DNA following DNA replication, while the endonuclease activity targets unmethylated target sites found on foreign DNA. Thus the complete enzyme can switch between the two activities. Each R subunit contains an ATP-hydrolysing DNA translocation motor domain and an endonuclease domain (26). Translocation does not involve DNA strand separation but rather tracks the DNA helical contour (21,27). The complete assembly prefers to methylate the hemi-methylated target sequences produced by DNA replication in the host bacterium and to cleave DNA containing un-methylated sequences found on invading DNA (28–30). Incomplete complexes such as $R_1M_2S_1$ can hydrolyze ATP and translocate but not cleave DNA (30) while M_2S_1 acts solely as a MTase (31). Despite these structural similarities, these Type I restriction systems are grouped into families, IA to IE, based upon DNA hybridization, subunit complementarity, antibody cross-reactivity and, to some extent, amino acid sequence similarity in M and R subunits. EcoKI and EcoBI are Type IA systems, EcoAI is a Type IB and EcoR124I is a Type IC (32,33).

The stability of the complete complex varies depending on the Type I restriction enzyme investigated. EcoKI is stable as $R_2M_2S_1$ (10), EcoR124I is stable as $R_1M_2S_1$ and binds the second R subunit with a dissociation constant of 240 nM (30) in the absence of cofactors and DNA, and EcoAI is stable as M_2S_1 with both R subunits binding weakly (29). Given the variation in structural forms, it is perhaps not surprising that a role for the reversible dissociation of R subunits in the restriction reaction has been proposed with the MTase core acting as loading protein for the R subunits (34). Indeed, it is noteworthy that early complementation experiments *in vitro* using mutated forms of EcoKI and EcoBI actually require such a role (35,36).

A clear conclusion from most analyses of Type I restriction enzymes is that to achieve full DNA cleavage one must have one complete complex per target site and this essentially means that the enzymes do not turnover. However this long held view (13,14,22) has recently been challenged in two papers by Bianco and colleagues (37,38). They have claimed to observe turnover of the EcoR124I Type I restriction enzyme in the cleavage reaction with a stable $R_2M_2S_1$ assembly of the enzyme (38) and furthermore that RecBCD will displace EcoR124I to assist it in turning over (37). In their experiments, they generally had an excess of DNA target sites over EcoR124I enzyme so turnover would indeed appear to be occurring. They claim that this observation of turn-over can be extended to all Type I restriction enzymes and that there is no role for reversible subunit dissociation in the restriction reaction. Given that much of the early information on turnover was achieved with partially purified enzyme preparations we revisit the question of turnover

and the influence of RecBCD on the EcoKI Type I restriction enzyme and its MTase core (referred to as M.EcoKI), and also examine the DNA structure around the target site ($AACN_6GTGC$ with methylation detection of adenine at the underlined bases) required to initiate the ATP-driven DNA translocation.

MATERIALS AND METHODS

Chemicals

ATP, SAM and EcoRI were purchased from New England Biolabs. All other reagents were purchased from Sigma-Aldrich. Solutions were made up in Milli-Q water (18.2 MΩcm^{-1}).

Escherichia coli strains

Escherichia coli JM109 was purchased from Promega. *Escherichia coli* NM1261 (r$^-$m$^-$) was a kind gift of Noreen Murray (Institute of Cell and Molecular Biology, University of Edinburgh). *Escherichia coli* SCK387 for the overexpression of RecBCD was kindly provided by Robert Court (Cancer Research UK, Clare Hall Laboratories).

Plasmid DNA and oligonucleotides

pBRsk1 (39) is an engineered version of pBR322 (4361 bp) in which one of the two EcoKI sites (4024–4036) has been removed by site-directed mutagenesis. pUC18K2 (2.7 kbp) is the same as pUC18 with a unique EcoKI restriction recognition site engineered between the XbaI and SmaI site of the polylinker region. The unmethylated plasmid DNA was prepared from *E. coli* NM1261 (DE3). pBP520 (encoding RecC) and pBP800 (encoding RecB and RecD) for RecBCD overexpression were kindly provided by Robert Court and Dale Wigley (Cancer Research UK, Clare Hall Laboratories). Oligonucleotides were obtained from ATDBio (Southampton, UK) and annealed by mixing in 1:1 ratios, heating to 95°C and slow cooling to room temperature. Annealing was checked by analytical hplc to assess that it was successful as described previously (40). Their sequences are given in Table 1.

Enzyme preparation

EcoKI restriction enzyme, R subunit and M.EcoKI MTase were purified to >95% homogeneity as described previously (10,31). Concentrations of M.EcoKI and R subunit were determined by UV–Vis absorption but as quantities were limited, the concentration of EcoKI was determined by comparing the degree of staining of M subunits and S subunits in preparations of EcoKI and M.EcoKI on Coomassie-blue stained SDS–PAGE gels. RecBCD enzyme was purified by a modification of procedures supplied by Robert Court and Dale Wigley (Cancer Research UK, Clare Hall Laboratories) and is described in the Supplementary Data

Agarose gel assays

Digests (50 μl volume) were prepared comprising 100 mM Tris-acetate pH 7.5, 100 mM magnesium acetate, 7 mM

Table 1. Oligonucleotide duplexes used in ATP hydrolysis assays and CD spectroscopy

Duplex name	Sequence
75	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
45	5'-TGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCG-3' 3'-ACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGC-5'
21	5'-GCCTAACCACGTGGTGCGTAC-3' 3'-CGGATTGGTGCACCACGCATG-5'
45-4	5'-TGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCG-3' 3'-ACAGATCTATAGCCGGATTGG CCACGCATGCTCGAGTCCGC-5'
45/21	5'-TGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCG-3' 3'-CGGATTGGTGCACCACGCATG-5'
21/45	5'-GCCTAACCACGTGGTGCGTAC-3' 3'-ACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGC-5'
75/21	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-CGGATTGGTGCACCACGCATG-5'
21/75	5'-GCCTAACCACGTGGTGCGTAC-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
49TL/47 BR	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTAC-3' 3'-CGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
47TR/49 BL	5'-GCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATG-5'
75/45	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-ACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGC-5'
45/75	5'-TGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCG-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
75/49BL	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATG-5'
75/47BR	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-CGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
49TL/75	5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCGTAC-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
47TR/75	5'-GCCTAACCACGTGGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3' 3'-GTATAGGTGTAGGCCACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGCGTACGGCATCGCGCC-5'
CD duplex	5'-CACGGGCCTAACGATATCGTGCGTACGAGC-3' 3'-GTGCCCGGATTGCTATAGCACGCATGCTCG-5'
CD gapped duplex	5'-CACGGGCCTAACGATATCGTGCGTACGAGC-3' 3'-GTGCCCGGATTGC GCACGCATGCTCG-5'

The EcoKI target sequence is underlined.

2-mercaptoethanol, 50 µg/ml bovine serum albumin, 2 mM ATP, 0.1 mM S-adenosylmethionine (SAM), 2.5 nM unmethylated pBRsk1 and various concentrations of EcoKI or M.EcoKI and purified R subunit. RecBCD was added to 10 nM, if required, and the concentrations of Tris–acetate and magnesium acetate were both reduced to 10 mM for assays with RecBCD. The reactions were initiated by addition of ATP and incubated for varying lengths of time at 37°C and quenched by incubation at 68°C for 10 min. Samples were mixed with gel loading buffer and loaded onto a 0.9% agarose gel and subjected to electrophoresis in TAE buffer (40 mM Tris–acetate, 1 mM EDTA) at 100 V/h. DNA was visualized using ethidium bromide (0.1 µg ml^{-1}) under UV illumination. A digital image of the gel was analysed using ImageJ software. All experiments were performed in triplicate. Two different preparations of EcoKI were used and gave very similar results.

ATPase assays

ATPase assays were performed as described by using a coupled enzyme assay (41) and an extinction coefficient of 6220 M^{-1}cm^{-1} for NADH absorption at 340 nm.

Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed as described in Supplementary Data.

RESULTS

Enzyme turnover

Enzyme:DNA ratio. We examined the amount of digestion of a closed, circular (CCC) plasmid containing a single EcoKI recognition sequence as a function of enzyme to target site ratio, Figure 1a. If there was turnover then even if [enzyme] < [DNA], one would expect all of the DNA to be eventually cleaved to a linear (L) form via an open circular intermediate (OC). Densitometry, Figure 1b, clearly showed that the amount cleaved by EcoKI at different enzyme to target site ratios was exactly that expected if each EcoKI could only perform a single restriction event on a single DNA molecule. For instance, if there were twice as many DNA sites as enzyme molecules, then only about half the DNA was cut.
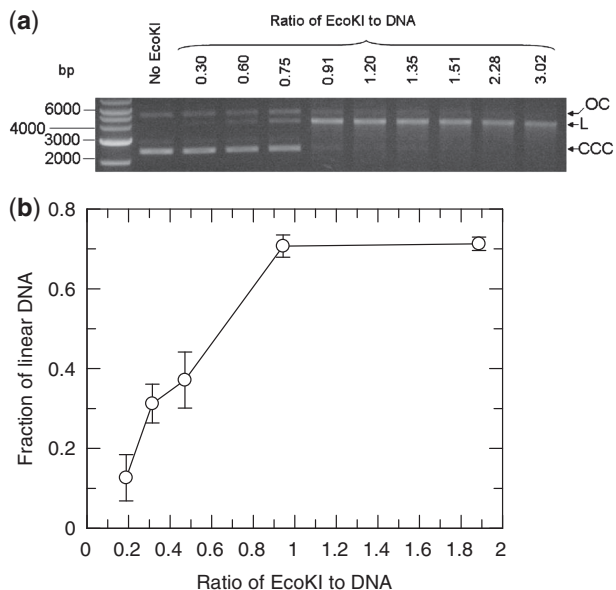
Figure 1. Digestion of 3 nM plasmid pBRsk1 with varying amounts of EcoKI. The reaction time was 10 min. (**a**) Agarose gel showing the conversion of closed circular (CCC) DNA via an open circular (OC) intermediate to a full length linear (L) product. The leftmost lane is a DNA size ladder. (**b**) Densitometric analysis of a series of agarose gels as in panel *a* showing the fraction of linear DNA obtained as a function of EcoKI:DNA ratio. Different preparations of EcoKI made on different dates were used in panels (**a**) and (**b**) but all of our preparations were comparable.

Extended reaction time. We also examined the kinetics of cleavage in this assay to ensure that the reactions shown in Figure 1 had gone to completion. A substoichiometric amount of EcoKI was used to digest pBRsk1 but the reaction time was increased up to 30 min, Figure 2. Reactions were performed in a single tube and aliquots withdrawn at specific times and quenched. Two different ratios of EcoKI to DNA were used and Figure 2 shows that the cleavage reaction reached an end point with the expected incomplete cleavage in ~10 min as there was no further increase in the amount of linear DNA after this time. Thus, the stoichiometric cleavage observed in Figure 1 was the maximum amount of cleavage possible for the given conditions.

Reconstitution of nuclease. Adding R subunit to M.EcoKI in various ratios and then performing a DNA digestion with enzyme in excess showed that cleavage only just started to become visible at a ratio of 1.5 R subunits to one M.EcoKI and complete cutting did not take place until a ratio of at least two R subunits to one M.EcoKI was attained, Figure 3. The absence of cutting when insufficient R subunits were present indicates that a stoichiometry of $R_1M_2S_1$ is not an endonuclease in agreement with earlier results on EcoKI (10) and on EcoR124I (30). Reconstitution of EcoKI restriction enzyme from M.EcoKI and R subunits does not lead to DNA cleavage unless sufficient R subunits are present to give a reasonable probability of forming $R_2M_2S_1$.

Effect of additional R subunits on activity of substoichiometric amounts of nuclease. Having demonstrated
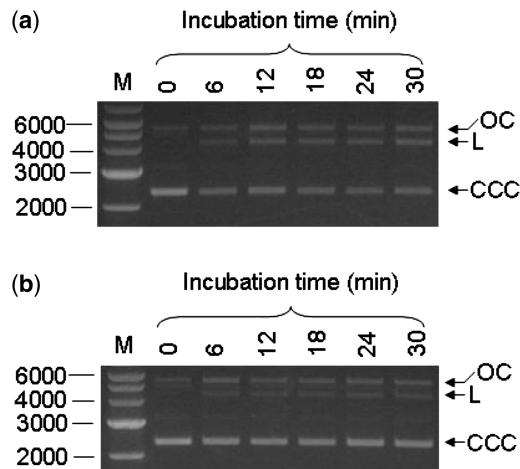

Figure 2. The effect of extended reaction times for ratios of EcoKI to DNA of 0.6:1 (**a**) and 0.4:1 (**b**). The concentration of plasmid pBRsk1 was 3 nM. The leftmost lane is a DNA size ladder as in Figure 1. The following lanes show time points of 0, 6, 12, 18, 24 and 30 min digestion. The reactions are complete within 6–12 min as no further increase in L DNA is seen at later times.
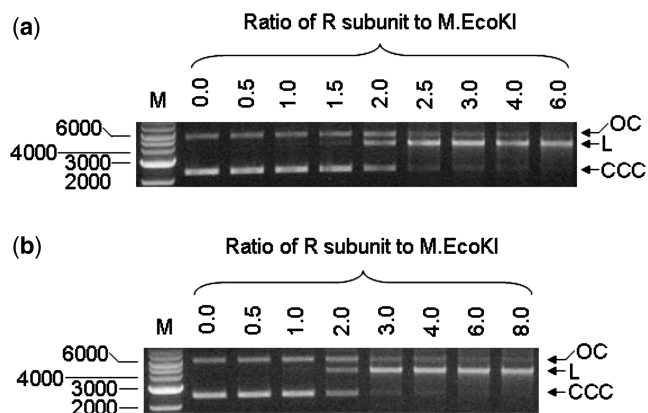

Figure 3. Reconstitution of EcoKI nuclease from purified M.EcoKI and R subunits using different ratios of R subunits to M.EcoKI. The nuclease was reconstituted as a 40× stock solution at room temperature for 5 min prior to adding to the reaction mix to initiate the reaction. The reconstituted nuclease was then used to digest unmethylated 3 nM pBRsk1 at 37°C for 8 min. The final concentration in the reaction of M.EcoKI was 7.5 nM with a varying amount of R subunit. The leftmost lane shows DNA size markers as in Figure 1, subsequent lanes show R subunit:M.EcoKI ratios of 0:1, 0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 4:1 and 6:1 in **a** and of 0:1, 0.5:1, 1:1, 2:1, 3:1, 4:1, 6:1 and 8:1 in **b**. Formation of L DNA is slight at a 1:1 ratio and only becomes obvious at 1.5:1 or greater ratios.

that DNA cleavage to completion requires one complete complex with two R subunits, we asked whether a stoichiometric amount of the MTase core (i.e. one core per site) was required for cleavage or whether the minimal requirement was just for two R subunits per site and less MTase could be present. In other words, could the MTase core be recycled? Figure 4 shows the partial digestion of DNA by a substoichiometric amount of the nuclease as expected from the results above. However, the further addition of purified R subunit to the partial digest so
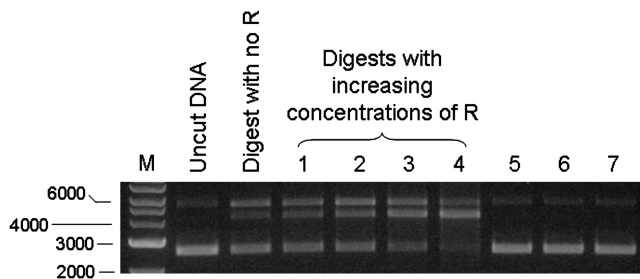
Figure 4. 3 nM pBRsk1 plasmid DNA was digested with a substoichiometric amount of nuclease (1 nM) in the presence/absence of different amounts of R subunit. The leftmost lane shows DNA size markers as in Figure 1. The next two lanes show uncut DNA and the partial cutting of the DNA when the ratio of nuclease to DNA was 1:3. The following lanes show the same ratio of nuclease to DNA of 1:3 but with additional R subunit being added. Ratio of nuclease to R subunit for Lanes 1–4 were 1:1, 1:3, 1:4 and 1:8, respectively. Lane 5, R subunit only. Lane 6, same as Lane 4 but no ATP. Lane 6, same as Lane 4 but no SAM.

Figure 5. Gel assay to test reactivation of EcoKI by RecBCD. Lane 1, undigested pBRskI (3 nM); Lane 2, partial digest of plasmid using EcoKI (1.5 nM) showing some L DNA; Lane 3, partial digest of plasmid by 1.5 nM EcoKI as in Lane 2 but in the presence of RecBCD (10 nM) showing the removal of the L DNA; Lane 4, after 5 min digestion an additional aliquot of plasmid DNA (3 nM) was added to the partial digest from Lane 2 and incubated for a further 5 min showing no further digestion but an increase in the amount of CCC DNA as expected; Lane 5, after 5 min digestion an additional aliquot of plasmid DNA (3 nM) was added to the partial digest performed in the presence of RecBCD (Lane 3) and incubated for a further 5 min showing the increase in CCC DNA but no extra digestion; Lane 6, after 5 min an additional aliquot of ATP (2 nM), SAM (0.1 mM) and plasmid DNA (3 nM) was added to the partial digest performed in the presence of RecBCD (Lane 3) and incubated for a further 5 min showing that sufficient cofactors were present.

that there were at least two R subunits per site, resulted in complete cleavage of the DNA. Thus it would appear during the restriction process on long DNA molecules, that R subunits can fall off from the core MTase of EcoKI and that the MTase core can be recycled to bind to more DNA and stimulate further cleavage by fresh R subunits. The corollary of this is that the R subunits do not get recycled and presumably remain on the DNA as suggested previously (15,19). The disassembly of EcoKI was neither observed during normal handling of the enzyme—it is a stable heteropentamer (10)—nor was it observed to fall apart during DNA footprinting experiments on short duplexes even when it was hydrolysing ATP (20). Hence, it is the length of the DNA supporting the ATP hydrolysis that is important to allow this recycling. Such a length dependence has been demonstrated previously for EcoBI (14,21).

Effect of RecBCD on EcoKI turnover. Bianco and Hurley (37) suggested that EcoR124I is displaced by the translocating RecBCD enzyme, resulting in restoration of its catalytic function. However, we have been unable to demonstrate a corresponding reactivation of EcoKI by RecBCD, Figure 5. We found conditions whereby the plasmid underwent partial digestion by limiting the amount of active EcoKI in the reaction mix. RecBCD was then added to the reaction mix to test whether the EcoKI would be displaced from the linearized DNA as this DNA is destroyed by the RecBCD. A displacement of EcoKI from DNA by the translocating RecBCD enzyme might reactivate the restriction enzyme allowing EcoKI digestion of the circular plasmid DNA to restart and go to completion. Digests were carried out in a low ionic strength buffer in which EcoKI and RecBCD were both active.

Addition of a substoichiometric amount of EcoKI relative to pBRsk1 resulted in partial cleavage of the DNA in 5 min in this low ionic strength reaction buffer (Figure 5, compare undigested sample in Lane 1 with partial EcoKI digest in Lane 2). The addition of 10 nM RecBCD in the reaction mix resulted in a complete loss of
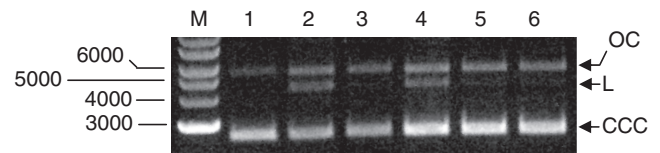
linearized plasmid DNA, but there was no apparent re-activation of EcoKI (Figure 5, Lane 3) as shown by the continued presence of closed circular and open circular DNA. After 5 min, an aliquot of the reaction mixture with and without RecBCD was removed and placed in a fresh tube containing 3 nM undigested pBRskI. As can be seen in Figure 5, Lanes 4 and 5, no further cleavage of the DNA was apparent even in the presence of RecBCD (Lane 5). To ensure that the lack of reactivation of EcoKI by RecBCD was not due to the cofactors being all used, additional ATP (2 mM) and SAM (0.1 mM) were also added to the reaction mix along with the add-itional pBRsk1 (Figure 5, Lane 6). The addition of a second differently sized plasmid, pUC18K2 instead of more pBSsk1, gave the same behaviour (Supplementary Figure S1). However, even in the presence of excess cofac-tors, no RecBCD-mediated reactivation of EcoKI could be detected as the freshly added circular DNA remained undigested. The halting of the restriction reaction of EcoKI by RecBCD agrees with earlier results using EcoBI (22).

DNA recognition sequence structural requirements for ATPase

We decided to examine the detailed structural require-ments of the DNA around the EcoKI recognition sequence in case these had an influence on the ATPase activity of the enzyme. A range of oligonucleotides were annealed to produce substrates containing a centrally located target sequence for EcoKI and various lengths of single- and double-stranded extensions. The core double-stranded sequence has been studied extensively for binding to EcoKI. In particular, footprinting studies showed even in the presence of ATP hydrolysis that all three types of subunit remained on the duplex (20) in contrast to the situation on long DNA molecules (15,16,19,36).

It is apparent from Figure 6 that a 21 bp duplex did not support significant ATP hydrolysis (0.04% of the
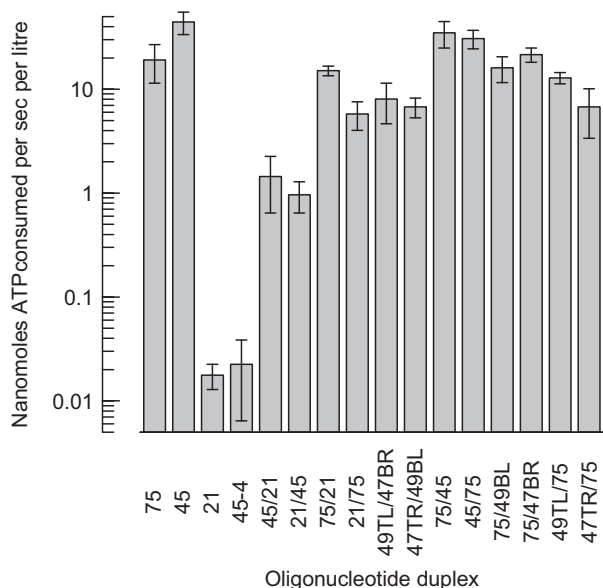
Figure 6. ATP hydrolysis activity of EcoKI on various oligonucleotides duplexes as measured using the NADH-coupled enzyme assay. The assay contained 10 nM EcoKI, 5 nM of the appropriate DNA duplex given in Table 1, 0.1 mM SAM and 2 mM ATP.

maximum observed) although it is known to bind well to EcoKI (42). This would be expected given that the MTase core of the enzyme would bind to this duplex and completely cover it preventing any contact with the ATP-hydrolysing R subunits. However, single-strand extensions on both sides of this 21 bp core duplex, so that the DNA was at least 45 bases in length, supported ATP hydrolysis to ~2.7% of the maximum observed. This 45 base length is slightly larger than the minimum footprint found for the EcoKI nuclease (20) so the single-strand extensions must be contacting the ATP-hydrolysing domains in the R subunit. Extending the single-strand regions to even greater lengths on both sides of the 21 bp core region gave a jump to ~20% of the maximum activity. Maximal activity was observed when the double-stranded region was increased in length from the 21 bp core to 45 bp and it made little difference if this double-stranded core had the EcoKI target sequence located centrally (duplexes 45, 75, 45/75 or 75/45) or was displaced to one side or the other of the target sequence (duplexes 75/49BL, 75/47BR, 49TL/75 or 47TR/75) as long as at least one of the R subunit ATP-hydrolysing domains was in contact with double-stranded DNA.

The action of RecBCD on an active complex of EcoKI on linear DNA would be to digest the entire duplex DNA back to the single region around the recognition site occupied by the EcoKI molecule. If this region were less than ~45 bp in extent, ATP hydrolysis would cease but the enzyme might remain bound to DNA. Early data showed such a cessation of ATP hydrolysis upon the action of RecBCD (22) rather than any reactivation as proposed by Bianco and Hurley (37).

We extended the analysis of ATP hydrolysis to oligonucleotide duplexes containing a short single-stranded region within the non-specific spacer part of the EcoKI recognition sequence. We have previously observed that such a single-strand gap facilitated tight binding by M.EcoKI but that the base flipping associated with enzyme activity was impaired and no methylation was observed (40). Using a 45 bp duplex as a standard with high ATPase activity, the introduction of the single-strand region within the non-specific spacer in the middle of the EcoKI target sequence (duplex 45-4) produced a dramatic reduction of ATPase activity to essentially undetectable levels. This result is rather surprising as the other ATPase data indicate that filling the R subunit ATP-hydrolysing domains with double-stranded DNA turns on hydrolysis. Clearly some DNA distortion in the central spacer region must be required to position the R subunits and the DNA outside the 21 bp core correctly for ATP hydrolysis.

To further investigate the nature of the distortion in the core 21 bp region, we recorded circular dichroism spectra of complexes of M.EcoKI and a normal duplex or a duplex containing the single-strand gap, Supplementary Figure S2. Protein binding caused an increase in the CD signal for normal DNA, as observed previously for the EcoR124I MTase (43), but the introduction of the gap prevented the change in CD despite the previous observation of good binding by M.EcoKI to this gapped duplex (40). The addition of SAM to the protein:DNA mixture produced no change in the spectra (data not shown). In summary, the introduction of the single-strand gap improves binding affinity but prevents base flipping, MTase activity (40), ATP hydrolysis, Figure 6 and DNA distortion, Supplementary Figure S2. Presumably the failure to form the correct protein:DNA conformation around the recognition sequence leads to further problems in forming contacts between R subunits and DNA. The loss of ATP hydrolysing ability is particularly intriguing.

DISCUSSION

Our data show that the stable $R_2M_2S_1$ EcoKI restriction enzyme does not turnover in the DNA cleavage reaction if this subunit ratio is strictly observed. It has a strict one enzyme per target site requirement in our assay. If this enzyme:site ratio is not achieved then only a fraction of the DNA can be cleaved. The amount cleaved is proportional to the amount of enzyme. The lack of turnover is not due to the methylation of the DNA target site as the EcoKI restriction enzyme has been shown to take many hours to produce even a minimal amount of methylation (44).

However, if excess R subunits are present then multiple cleavage events can take place. Therefore, the requirements for EcoKI to cleave DNA are to have two R subunits per DNA site and at least two R subunits per MTase as found for other Type I enzymes such as EcoAI (29) and EcoR124I (30). Excess MTase with insufficient R subunit (less than two R per MTase) leads to the formation of the inactive $R_1M_2S_1$ complex. These results now bring EcoKI, and presumably other Type IA restriction

enzymes such as EcoBI, closer in behaviour to the Type IB and IC restriction enzymes, which can use subunit dissociation and reassociation in their reaction cycle. The difference between the enzymes is merely one of the stability of the active $R_2M_2S_1$ complex in the absence of DNA. The R subunit can be considered to be the trigger for restriction and the MTase has the status of a 'loading' protein responsible for targeting the R subunits to act on DNA containing unmethylated targets.

We have observed that although RecBCD can easily degrade the EcoKI–DNA complex after a DNA end has been revealed by the EcoKI endonuclease activity, the EcoKI $R_2M_2S_1$ molecule appears to be incapable of attacking further DNA molecules presumably because it has a short duplex containing the recognition sequence still bound. In other words, RecBCD does not make EcoKI turnover in its restriction endonuclease function in agreement with older observations in which RecBCD and DNase brought DNA cleavage and ATP hydrolysis by the related EcoBI enzyme to an abrupt halt (22). We understand that the EcoR124I enzyme also cannot be reactivated by RecBCD (Mark D. Szczelkun, personal communication) in contrast to claims made by Bianco and Hurley (37). We cannot explain this discrepancy.

Bianco *et al.* (38) have proposed that all Type I restriction enzymes are able to turnover without dissociation and reassociation of subunits, in other words, that the Type I restriction enzymes are stable entities with stoichiometry $R_2M_2S_1$ throughout the restriction process and are 'true enzymes'. This contradicts much published evidence for EcoR124I (30) and EcoAI (29) Type I enzymes and now we have demonstrated a role for dissociation and reassociation for EcoKI in the restriction reaction. We wish to put forward some suggestions about the origin of this difference between the work of Bianco and Hurley (37) and Bianco *et al.* (38) and other groups even though we ourselves have rarely worked with the EcoR124I system. Other studies have always purified predominantly the $R_1M_2S_1$ form of EcoR124I and additional R subunits have had to be added exogeneously to obtain maximum DNA cleavage ability (30,34). A similar requirement has been found for the Type IB EcoAI enzyme *in vitro* (29). The purification method used by Bianco and colleagues (37,38), although somewhat different from other methods of purifying EcoR124I, produces a product as well-defined as the other methods, namely, a single chromatographic peak containing R, M and S subunits when analysed via SDS–PAGE. They have assumed that their protein has a stoichiometry of $R_2M_2S_1$ (37,38). They also present an elution profile of their pure protein eluting from a denaturing gel filtration column. Since the sequence and UV-absorption extinction coefficients are known for each subunit in EcoR124I, it is simple to calculate the relative proportion of the R subunits to the MTase core (the M and S subunits overlap in the elution profile). Using their data, we calculate that their purified EcoR124I protein has a stoichiometry of $R_{1.2}M_2S_1$, a non-integer stoichiometry, and hence is a mixture containing one complete $R_2M_2S_1$ enzyme for every four partially assembled $R_1M_2S_1$ complexes. Both of these complexes will hydrolyze ATP but only the first will

cut DNA accounting for their low specific activity. (Given their assumption of 100% $R_2M_2S_1$ this also means that their protein concentrations are slightly incorrect but this is not too significant.) Since they have $R_1M_2S_1$ complexes, redistribution of R subunits between complexes during measurements of DNA cleavage must therefore serve to explain their results and would bring their work in line with that of others working on EcoR124I particularly those employing single molecule methods where the action of individual R subunits in $R_1M_2S_1$ and $R_2M_2S_1$ complexes can be detected during translocation and cleavage (34). Having said this, Bianco *et al.* (38) do present data indicating turnover of these dissociating/reassociating EcoR124I complexes as they demonstrate cleavage when DNA is definitely in considerable excess of EcoR124I. We have not been able to demonstrate this with EcoKI so there is a difference between the two enzymes and it must rest presumably with the nature of the structure of the enzyme:DNA complex at the point of cleavage with the EcoR124I R subunits falling off the DNA ends easily and the R subunits of EcoKI staying bound for a longer time as suggested previously (15).

We have also examined the requirements for ATP hydrolysis to be initiated by EcoKI using a range of variations of the recognition site structure guided by previous DNA footprinting experiments (20,42). While a minimum size of 21 bp containing the recognition sequence is sufficient for binding, it is not sufficient for ATP hydrolysis as might be expected from the larger footprint of the complete RM enzyme. ATPase activity requires the presence of the 21 bp region plus single-strand extensions of around 12 bases on either side of the 21 bp region. It does not matter whether these extensions are actually double stranded, although this enhances the ATPase activity, nor does it matter whether the single strands are in a 5′ to 3′ orientation or 3′ to 5′ orientation. However, the introduction of a gap in the non-specific spacer sequence in the middle of the recognition sequence results in complete loss of ATPase activity. It has previously been shown that the gap removed the ability of the enzyme to base flip and methylate the target adenines (40) and this result is now supported by CD spectroscopy. The complete double-stranded recognition sequence becomes distorted upon EcoKI MTase binding but this signal and distortion is lost when the recognition sequence contains a gap. A change in CD signal from DNA containing the recognition sequence has been observed for the complex of DNA with EcoR124I MTase (43). Thus a complete double-stranded recognition sequence (13 bp) including a bend and base flipping, double-stranded regions of ∼4 bp on either side (21 bp total) and further single- or double-stranded regions adding up to the footprint of EcoKI are required for the DNA translocase motors in each R subunit to start ATP hydrolysis. On the short duplexes examined in this work and in footprinting studies (20,42), the R subunits remain attached to the MTase core futilely hydrolysing ATP but unable to translocate DNA. The results with the gapped duplex show that fully double-stranded DNA within the core MTase is essential for the R subunits to hydrolyze ATP. This implies that the stiffness of the DNA duplex

and its resistance to twisting in the MTase core is somehow involved in potentiating ATP hydrolysis by the R subunit. On longer DNA molecules, translocation can commence and it would appear that the contacts of the R subunits to the MTase core become unstable. Since single molecule studies clearly show the formation of loops by EcoKI and other Type I RM enzymes during translocation, the R subunits do not dissociate from the MTase until either too much tension has been generated in the DNA or DNA cleavage takes place (15–17). Given that ATP hydrolysis continues long after cleavage has been achieved, perhaps the R subunits reassociate with the MTase core and start translocating once more or remain bound at the cleavage site but releasing the MTase core to be re-used in initiating a further round of cleavage (15,17,21,25). The nature of the R subunit to DNA contacts at the cleavage site still needs to be defined.

Having analysed our data and compared it to the results of others with the conclusion that EcoKI uses a subunit dissociation/reassociation mechanism as found for other Type I restriction enzymes, we would like to examine three more general questions.

 (i) Type I restriction enzymes can hydrolyze vast amounts of ATP *in vitro*. Is this a problem *in vivo* as suggested (14,22)?
 (ii) Do Type I restriction enzymes, or indeed any restriction enzymes, need to have evolved the ability to turnover?
(iii) A fraction of invading DNA molecules, whether from phage or plasmids always evade the Type I restriction reaction and are subsequently found to be methylated by the M_2S_1 core of the Type I RM system. How does this happen in any reasonable physiological time when *in vitro* studies of the Type I restriction enzymes show that they are appallingly poor MTases when operating on unmethylated DNA (29,30,43–45)?

The observations of extensive ATP hydrolysis during and for long after the cleavage reaction suggested that if this occurred within a living cell, then there would be such a severe depletion of intracellular ATP concentration that the cell would die (14,22). Data on *E. coli* have grown hugely since this suggestion. It has been estimated that 5.5×10^{10} molecules of ATP are used to make a single *E. coli* cell in about 30 min or it uses 3×10^7 ATP per second (46). Obviously ATP synthesis must at least equal this or exceed this amount. It has also been estimated that there are about 60 molecules of EcoKI per cell (47) that can translocate at $\sim$1000 bp/s using $\sim$1 ATP per base pair translocated (21,34). Thus even if all the EcoKI in a cell were hydrolysing ATP as fast as they could (6×10^4/s), the burden on the cell would be only 0.2% of the total ATP and we suggest that this is insignificant. The action of exonucleases like RecBCD in halting ATP hydrolysis and DNA cleavage by the Type I restriction enzyme, demonstrated clearly *in vitro* (22), would reduce the burden still further. However, should the cell run short of ATP, alleviation of restriction would start due to the cell becoming stressed and it

would ultimately survive (47,48). Thus it would seem that this problem can now be put to rest.

Do restriction enzymes need to turnover? Given that most bacterial cells will be unlikely to be attacked by phage or other mobile genetic element in any given cell generation and that only a single attack is most probable unless the cell is colonizing a new environment (49), then there will be more than sufficient numbers of EcoKI (about 60 per cell) (47) or indeed any other restriction enzyme, including the properly catalytic Type II restriction enzymes which do exhibit turnover, to tackle any attack without requiring multiple turnovers of a single restriction enzyme. The apparent waste of energy in synthesizing a large Type I restriction enzyme for a single double-stranded cleavage, when a smaller Type II restriction enzyme can achieve the same effect, is not so extreme when only a single turnover is required. Thus the ability to turnover in the restriction reaction is almost certainly not an evolutionary requirement for a restriction enzyme although it is of considerable use in the molecular biology laboratory.

Lastly, we feel that the important question on the activity of Type I RM systems is not whether they turnover in restriction but rather how they methylate DNA containing unmethylated target sequences. Following a phage lambda infection for example, a fraction of phage survive the attack of the restriction enzyme and, if modification is not blocked in some manner by the phage (e.g. the ocr system of phage T7 prevents modification) (50), then the surviving phage become methylated by the modification MTase part of the RM system. Most MTases from the Type II RM systems are roughly equally efficient at methylating unmethylated target sequences and unmethylated target sequences, i.e. they are *de novo* MTases (51). However, EcoKI, EcoAI and EcoR124I are extremely poor *de novo* MTases but good maintenance MTases (i.e. they prefer hemimethylated targets on the host DNA, 29,30,43). Methylation by EcoKI of unmethylated targets *in vitro* never appears to go to completion even in 24 h at which point only a small percentage of the DNA is methylated (31,44,45). This completely contradicts the *in vivo* observations where, for example, an infection by a single phage lambda produces a burst of 50–100 new phage each with five fully methylated EcoKI target sites in the order of 60 min (47,52). Given that EcoKI would far rather cleave unmodified DNA (of order one in 10^4 or 10^5 phage escape restriction to become modified) than methylate it, it seems extraordinary that full methylation can occur. Phage lambda has an open reading frame encoding the Ral protein (53) and some *E. coli* strains contain the Rac prophage encoding the Lar protein (54). Removal of Lar from *E. coli* enhanced the effectiveness of restriction by the EcoKI RM system (54,55). It is believed that these proteins enhance the *de novo* MTase activity of EcoKI but the mechanism is unknown. *De novo* MTase activity can also be obtained for EcoKI by mutations in the M subunit but the mechanism is probably unlike that used by Ral and Lar (56). Given the presence of Type I RM systems in >50% of bacteria (57), mechanisms involving

functions such as *ral* and *lar* must be quite common if mobile elements are to survive restriction and become methylated. More research is required on these anti-restriction functions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

FUNDING

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