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## Routes of DNA Cleavage by Type II Restriction Enzymes

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### ABSTRACT

The aim of this review is documentation of all the possible cleavage mechanisms of restriction endonucleases. The orthodox restriction endonucleases, which are widely used in molecular biology, recognize and cleave DNA at a single palindromic DNA recognition sequence. They show a high sequence and structural diversity. There are several indications about their convergent evolution from different protein families. Hence it is expected that they must show a great diversity in the DNA cleavage reaction mechanisms. They have been divided into subgroups as Type IIP, E, F and S according to their subunit structure and required number of the cleavage sites, but this classification still found to be insufficient due to diversity in the reaction mechanisms among the group members. In this article, a new classification scheme of orthodox restriction enzyme, based on their choice of phosphodiester bond cleavage is presented. A restriction enzyme may bind to one- or two recognition sites thus making available two or four target phosphodiester bonds to its catalytical site/s. Difference in the cleavage of these available phosphodiester bonds have been used as the basis of classification of restriction enzymes. The restriction enzymes have been classified in six groups viz., 1 of 2, 2 of 2, 1 of 4, 1+1 of 4, 2 of 4 and 4 of 4, where, former figure indicates number of cleaved phosphodiester bond and the later is number available phosphodiester bonds.

**Key words:** Endonuclease, reaction mechanism, enzyme, enzyme kinetics, plasmid

### INTRODUCTION

Restriction endonucleases or restriction enzymes (REases) are well known as molecular scissors for their indispensable use in molecular biology and gene cloning (Pingoud *et al.*, 1993). However, in biological systems, the restriction enzymes co-exist with their counterpart DNA methyltransferase and constitute together Restriction-Modification (RM) systems (Roberts *et al.*, 2010; Watanabe *et al.*, 2006) that provide defense to the host against invading phages (Arber and Linn, 1969). Sometimes RM systems have been often found to behave as mobile genetic elements (Furuta *et al.*, 2010; Kobayashi, 2004). Restriction-modification enzymes have been classified into subtypes I, II, III and IV based on their subunit organization, cofactor requirement, target sequence and cleavage position (Bickle and Kruger, 1993; Roberts *et al.*, 2003). Among these subtypes, type II restriction enzymes secure the most important place in application as they cleave DNA at specific sequences, usually palindromes 4-8 bp long, in a reaction that requires  $Mg^{2+}$  as a cofactor (Roberts *et al.*, 2010). Type II restriction enzymes are further classified into type IIP, IIE, IIF and IIS (Mucke *et al.*, 2003).

Type IIP restriction enzymes, also known as orthodox type II restriction enzymes, are dimeric proteins that interact symmetrically with their palindromic targets, in such a way that one catalytic site in the dimer is positioned to cleave the target phosphodiester bond of one strand of the DNA and the other site is on the equivalent bond in the complementary strand (Aggarwal, 1995; Ishikawa *et al.*, 2005; Miyazono *et al.*, 2007). Type IIE and IIF enzymes exhibit the specific property that they have to simultaneously bind two copies of their DNA recognition sequences to cleave the DNA (Mucke *et al.*, 2003).

Type IIE restriction enzymes exist as homodimer in the solution and are capable of binding to two equivalent recognition sites on same DNA molecule (in cis) or on two different molecules (in trans) (Conrad and Topal, 1989; Kruger *et al.*, 1988; Pein *et al.*, 1991), where one site works as allosteric effector and the other as substrate (Colandene and Topal, 1998; Reuter *et al.*, 1999). However, the cleavage rate for in cis reactions (two-site plasmids) is much higher than in trans (one-site plasmids). They contain two domains, as seen in crystal structure of EcoRII and NaeI, a DNA binding domain and an endonuclease like catalytical domain (Golovenko *et al.*, 2009; Huai *et al.*, 2000; Zhou *et al.*, 2004). The binding domain of both the dimer binds with one copy of recognition site and the catalytical domain aligned to the target phosphodiester bonds in the substrate site. The binding to such two sites in EcoRII and NaeI showed positive cooperativity (Gabbara and Bhagwat, 1992; Kupper *et al.*, 1995; Yang and Topal, 1992). The binding domain of EcoRII has been constructed and purified separately, which showed slightly different binding constants ( $K_D$ ) for DNA as compare to the native enzyme, whereas the isolated catalytic domain binds very poorly to DNA (Golovenko *et al.*, 2009; Mucke *et al.*, 2002). However, the truncated C-terminal endonuclease like domain forms a dimer and cleaves a single-site DNA more rapidly as compare to native enzyme (Mucke *et al.*, 2002).

Type IIF restriction enzymes are homotetrameric as reported in crystal structures (Bozic *et al.*, 1996; Deibert *et al.*, 2000) and gel filtration chromatography (Khan *et al.*, 2010). They recognize and bind to two copies of their recognition site and cleave both of them together in concerted manner. In the crystal structure of some type IIF enzymes such as Cfr10I (Bozic *et al.*, 1996), NgoMIV (Deibert *et al.*, 2000) and Bse634I (Grazulis *et al.*, 2002), it was observed that two subunits form one of the equivalent DNA binding sites and two such dimers pack back-to-back to constitute an active tetramer. The active complex consists of the tetrameric enzyme bound with two copies of their DNA recognition sites. This feature of type IIF enzyme is comparable with type IIE that consists of a dimer and two copies of the sites. However, in type IIF enzymes both the active sites of the enzyme are identical. The kinetic studies carried out for one- and two-site plasmids for these enzymes revealed that they concertedly cleave both sites together, thus releasing the final product; and cleavage rate for the two-site plasmid is considerably higher than the one-site plasmids (Khan *et al.*, 2010; Siksnys *et al.*, 1999; Wentzell *et al.*, 1995). In SfiI, the communication between the two DNA binding sites was examined by disrupting one of the polar interaction in the hydrophobic interface between the two back-to-back dimers. The tyrosine residue was mutated to phenylalanine. Although, the enzyme remains as tetrameric in the solution but interestingly it showed maximal activity when bound to single site and had lower activity when bound to double sites (Bellamy *et al.*, 2007).

Type IIS restriction enzymes, such as FokI, bind to DNA in the monomeric states. This DNA-monomer complex, after interacting with another identical complex, forms a dimeric assembly with two DNA sites, which cleaves the DNA. Such reactions usually involve formation of a DNA loop (Catto *et al.*, 2006, 2008). Their DNA recognition site is asymmetric and the

Table 1: Expected reaction rate (k)\* for different substrates

Reactions	Substrate					
	1 of 2	2 of 2	1 of 4	1+1 of 4	2 of 4	4 of 4
One-site plasmid	$k_1 > k_2$	$k_2 > k_1$	$k_1 > k_2$	$k_1 > k_2$	$k_2 > k_1$	$k_2 > k_1$
Two-site plasmid	$k_3 > k_4 = k_5$	$k_4 = k_5 > k_3$	$k_3 > k_4 > k_5$	$k_3 > k_5 > k_4$	$k_4 > k_5 > k_3$	$k_5 > k_4 > k_3$
One-site vs. two-site	$k_2 = k_4 = k_5$	$k_2 = k_4 = k_5$	$k_2 < k_4 > k_5$	$k_2 < k_5 > k_4$	$k_2 < k_4 > k_5$	$k_2 < k_5 > k_4$

cleavage position is definably located downstream (Szybalski *et al.*, 1991). These enzymes have two domain structures, as found in FokI, a sequence specific N-terminal DNA binding domain and a sequence-unspecific catalytic domain (Li *et al.*, 1992). In solution, the monomeric FokI can bind to the DNA but can not cleave it, as single catalytic domain is incapable to cleave both the DNA strands and the subsequent dimerization become essential (Bitinaite *et al.*, 1998; Sanders *et al.*, 2009; Wah *et al.*, 1998).

Type II restriction enzymes have shown several diverse reaction mechanisms to cleave DNA (Embleton *et al.*, 2001; Gowers *et al.*, 2004; Khan *et al.*, 2010), besides these, there are still several possibilities, not explored yet. This article will provide summary of the possible reactions mechanism of type II restriction enzymes.

## MATERIALS AND METHODS

Arbitrary reaction rates, according to Table 1, were fitted to Eq. 1 to generate the kinetic graphs using GraphPad Prism V4.0.

$$d[P]/d[t]=k[S] \quad (1)$$

where, [S] is the concentration of supercoiled plasmid and [P] is the concentration of the various products (see section Experimental design). For the reaction boost in the presence of oligoduplex, a ten fold increase in reaction rate was assumed.

## RESULTS AND DISCUSSION

**Experimental design:** The reaction profile of a type II restriction enzyme with two sets of specially designed plasmids, one with a single copy of its recognition site and the other with two copies, will provide a diagnostic assay to identify its mode of DNA cleavage (Bilcock *et al.*, 1999; Embleton *et al.*, 2001). Cleavage of both the phosphodiester bonds of the DNA will generate the linear fragment, whereas cleavage of the single bond will result in nicking of the DNA. The nicking of the supercoiled plasmid (SC), by relaxing the superhelicity, will result in the production of the Open Circular (OC) DNA (Fig. 1). The plasmid with one target site (one-site) will convert to the OC form after nicking and subsequent cleavage of the second phosphodiester bond will result in the full length linear DNA (FLL). On the other hand, in a two-site SC plasmid, nicking of any of the four available phosphodiester bonds will result in its conversion to the OC form. Subsequent cleavage of the opposite phosphodiester bond will produce the FLL DNA and successive cleavage of remaining two phosphodiester bonds will release two linear fragments (L1 and L2) (Fig. 1). However, double nicking of a two-site plasmid will results in a doubly-nicked OC plasmid which will not be easily distinguished from single nicked OC DNA. Various forms of these products can be resolved and quantitated in an agarose gel electrophoresis (Khan *et al.*, 2010).

In another set of experiments, a DNA oligonucleotide duplex with one recognition site will be added to the reaction mixture with the one-site plasmid. This DNA fragment will facilitate in trans

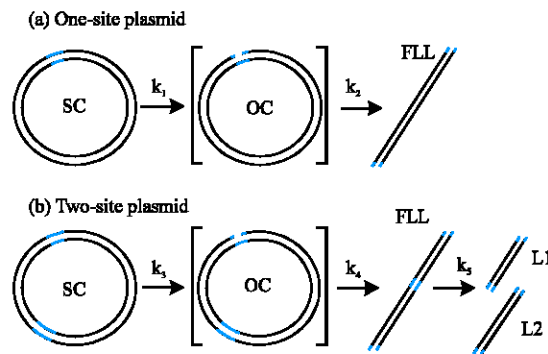


Fig. 1: Reaction schemes of a type II restriction enzyme at its recognition sites (shown in blue color) on a double-stranded DNA (depicted as parallel lines). (a) A supercoiled (SC) DNA with one recognition site forms the open-circle (OC) DNA by nicking of single strand and finally forms completely cut full-length linear DNA (FLL). (b) An SC DNA with two sites forms the OC by nicking of single strand, which will give FLL DNA by complete cleavage of one site; successive cleavage of the remaining site will give two linear DNA fragments L1 and L2 ( $k$  is the respective cleavage rates)

reactions occurring bridging of two reaction sites present on two different DNA molecules. Addition of the oligoduplex on enzyme requiring one site will not show any detectable difference in the reaction rate, but it will drastically enhance cleavage rate for the enzyme requiring two sites (Wentzell *et al.*, 1995).

### Various forms of reaction mechanisms

**1 of 2 cleavage:** In this type of reaction mechanism, the enzyme interacts with a single recognition site and cleaves only one out of two available phosphodiester bonds before dissociating from the substrate. Initially, reaction will result in accumulation of OC DNA from both one- and two-sites substrates (Fig. 2a). In the steady-state condition, the initial cleavage rate for OC DNA will be higher than FLL DNA (Table 1). However, the substrate consumption rate will be the same in a one- and two-site plasmids. Addition of the oligoduplex will not cause any effect on the cleavage of one site plasmid. This type of reaction mechanism has been reported for KasI (Gowers *et al.*, 2004).

**2 of 2 cleavage:** A restriction enzyme showing this mechanism behaves as a true orthodox type IIP enzyme. The enzyme interacts only with a single recognition site and cleaves both the available phosphodiester bonds before leaving the substrates. Hence, each binding event results the in complete cleavage and the OC form is generated only as a small fraction formed by some rare incomplete reactions (Fig. 2b). Each cleavage reaction is accompanied by an individual binding event; hence substrate consumption rate for one- and two-site plasmids will be similar (Table 1). Addition of the oligonucleotide will not cause any effect on cleavage of one site plasmid. Most of the type IIP restriction enzymes show this type of reaction mechanism (Gowers *et al.*, 2004; Roberts *et al.*, 2010).

**1 of 4 cleavage:** This type of restriction enzyme recognizes and binds to two copies of their recognition sequence. The enzyme will cleave only one of the four available phosphodiester bonds; hence, in initial phase of reaction, OC DNA will supercede all other forms (Fig. 3a). In one-site

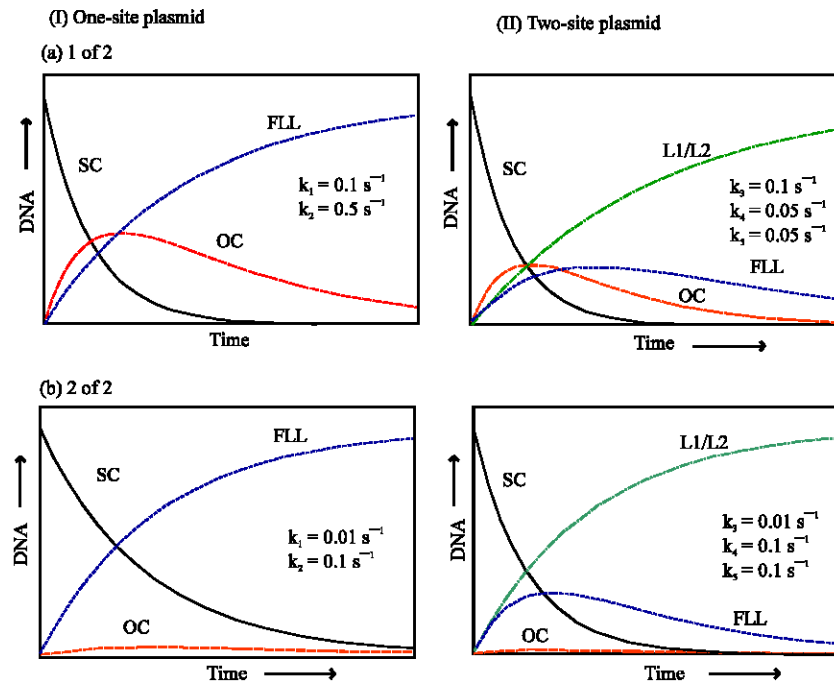


Fig. 2: Cleavage kinetics of a type II restriction enzyme characterizing (a) 1 of 2 and (b) 2 of 2 reaction mechanisms on (I) one-site supercoiled (SC) plasmid, (II) two-site supercoiled plasmid. The supercoiled (SC) DNA may give rise to open-circular (OC) , full-length linear (FLL) and linear fragments (L1 and L2)

plasmid, substrate consumption rate will be much less than the two-site substrate, substantiating requirement of two sites for efficient cleavage (Table 1). The one-site plasmid will be cleaved only in trans reactions occurring on two recognition sites on two different DNA molecules. The in trans reactions are intrinsically disfavored due to steric hindrance between the two supercoiled DNA molecules, in comparison to in cis reactions, where both the recognition sites are present on the same molecule (Wentzell *et al.*, 1995). Each reaction will be followed by the complete dissociation of the enzyme-substrate complex and the enzyme will search again for the binding sites. Hence, the substrate cleavage will proceed by multiple turnovers, resulting in drastically low rate of final product release. Addition of an oligoduplex will increase the efficiency of *in trans* reactions leading to increased substrate consumption rate (Fig. 3a, III). This type of reaction mechanism has been observed in NarI (Gowers *et al.*, 2004).

**1+1 of 4 cleavage:** These enzymes also recognize and bind to two copies of their recognition sites. The enzyme will cleave only one phosphodiester bond of each site, hence total two bonds will be cleaved at two different sites. This is comparable to 2 of 4 cleavage, where both the cleaved bonds are in the same site. The stringent requirement of two sites will result in slow cleavage rate of one-site plasmid (Fig. 3b, I and Table 1). The two-site plasmid will generate OC DNA in the first turnover that will be converted to completely cleaved products (L1 and L2) in subsequent turnovers. Production of the FLL fragment will be limited and occur only through in trans reactions. Addition of the oligoduplex will increase only rate of the nicking in one-site plasmid but reaction will not proceed to complete, resulting in production of OC DNA only. However in some

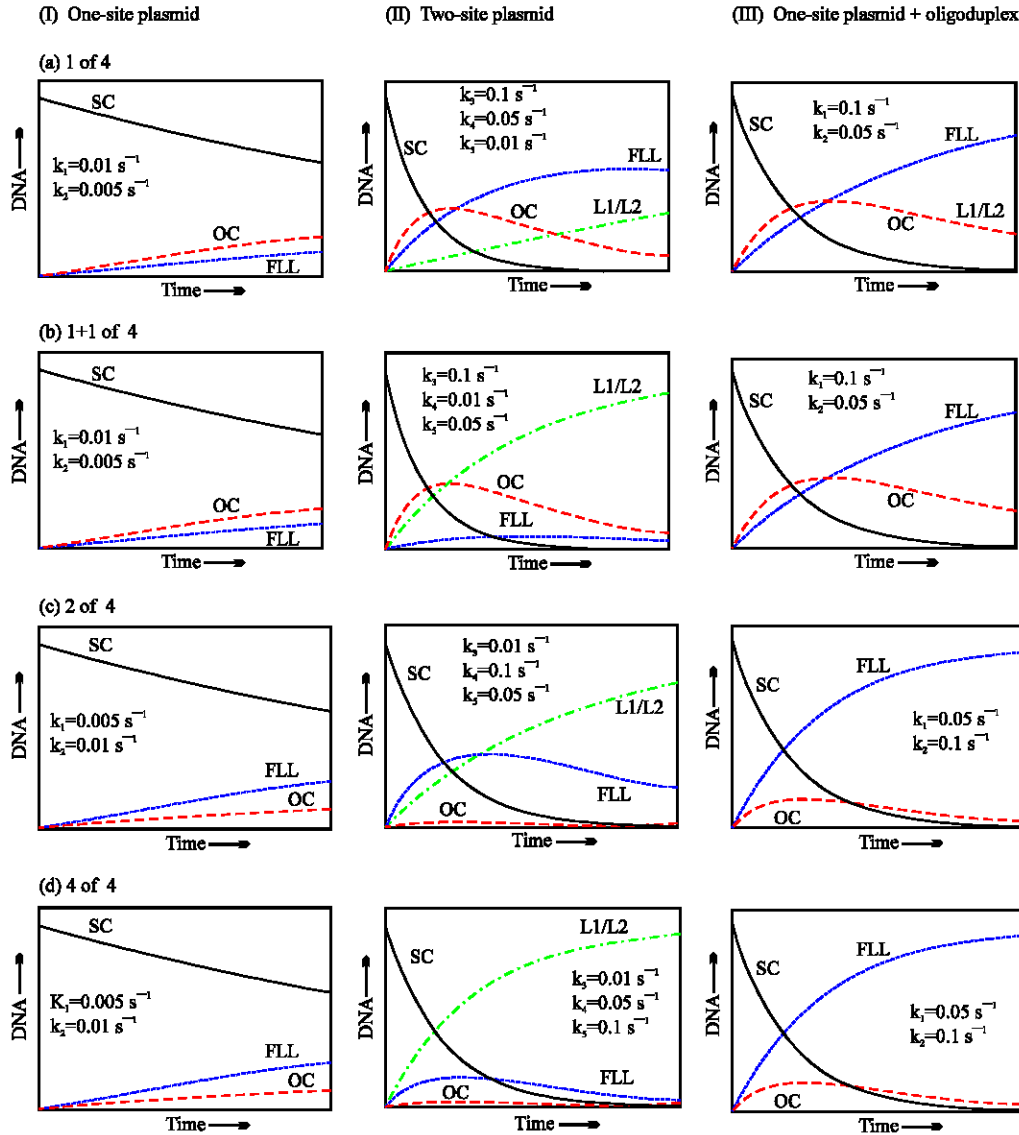


Fig. 3: Cleavage kinetics of a Type II restriction enzyme characterizing (a) 1 of 4, (b) 1+1 of 4, (c) 2 of 4 and (d) 4 of 4 reaction mechanisms on (I) one-site supercoiled (SC) plasmid (II), two-site supercoiled plasmid and (III) the one-site supercoiled plasmid in the presence of the oligoduplex. The supercoiled (SC) DNA may give rise to open-circular (OC), full length linear (FLL) and linear fragments (L1 and L2)

cases, as seen in PluTI (Khan *et al.*, 2010), the enzyme may release the substrate partially and rebound. In case of two-site substrates, binding to the released site may be more probable and most of the reactions may result in fully cut linear fragments L1 and L2. The cleavage kinetics for a two-site substrate will appear same as 4 of 4 cleavage, but will be different in one-site plasmid with and without oligoduplex; and in both the cases, OC product will supercede FLL form (Fig. 3b, III).

**2 of 4 cleavage:** These enzymes are broadly defined as type IIE. They bind to two sites but cleave only one and the other site merely acts as an allosteric effector. The two phosphodiester bonds of

the same site will be cleaved together resulting in linearization of the SC plasmid (Fig. 3c). The OC DNA will be produced rarely by incomplete reactions occurring *in trans*. Reaction on the one-site substrate will occur only in trans and stringent requirement of the two sites will result in considerably lower cleavage rate (Fig. 3c, I). In contrast, the two-site plasmid will serve as an efficient substrate and will be cleaved efficiently but only at one site. Cleavage of the remaining site will proceed at lower rate by in trans reactions (Fig. 3c, II and Table 1). Addition of an oligoduplex with one-site plasmid will increase the efficiency of the in trans reactions leading to increase in substrate cleavage rate (Fig. 3c, III). This type of reaction mechanisms have been observed in NaeI (Embleton *et al.*, 2001) and EcoRII (Piatrauskene *et al.*, 1996).

**4 of 4 cleavage:** These types of restriction enzymes are defined as type IIF. They efficiently bind and cleave all four phosphodiester bonds, in a two-site substrate, in a concerted manner. However, cleavage of the one-site substrate is considerably slower than the two-site substrate indicating requirement of two sites (Table 1). Reactions on one-site plasmid will occur only in trans and will lead to complete cleavage of the substrate, hence OC DNA will be produced only in minute amounts (Fig. 3d, I). On the other hand, complete cleavage of the two-site substrate will result in release of completely cut products (L1 and L2); the FLL and OC DNA will be produced in minute quantities by reactions occurring in trans (Fig. 3d, II). Addition of the oligoduplex, in the reaction upon one-site substrates, will enhance cleavage rate of one-site substrates (Fig. 3, dIII). This type of reaction has been observed in Mly113I (Gowers *et al.*, 2004) and Cfr10I (Embleton *et al.*, 2001).

## CONCLUSIONS

Cleavage pattern of several linear DNAs, plasmids and catenanes can provide a greater insight about cleavage mechanisms of a restriction enzyme. Reaction kinetics will also depend on the cleavage mechanism as observed in a two-site requiring restriction enzyme PluTI, where  $V_{\max}$  is 27-fold higher for two-site substrate compare to one-site substrate but similar in case of one- and two-site linear substrates (Khan *et al.*, 2010). This data substantiated the role of superhelicity in the cleavage reactions. Superhelicity facilitates only *in cis* reactions and hinders *in trans* reactions even in the absence of any apparent steric constraints.

Restriction enzymes constitute a group of structurally and evolutionary diverse proteins (Orlowski and Bujnicki, 2008; Roberts *et al.*, 2003), hence diversity in the reaction mechanism can be expected. Sometimes, one enzyme can show different mechanisms depending reaction conditions such as buffer, salt (Gowers *et al.*, 2004) and temperature. Hence, the above mentioned six mechanisms can not be considered inclusive for all restriction enzymes and exceptions can be expected.

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