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Review

Type II restriction endonucleases: structure and mechanism

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Abstract. Type II restriction endonucleases are components of restriction modification systems that protect bacteria and archaea against invading foreign DNA. Most are homodimeric or tetrameric enzymes that cleave DNA at defined sites of 4–8 bp in length and require Mg^{2+} ions for catalysis. They differ in the details of the recognition process and the mode of cleavage, indicators that these enzymes are more diverse than originally thought. Still, most of them have a similar structural core and seem to share a common mechanism of DNA cleavage, suggesting that they evolved from a common ancestor. Only a few restriction endonucleases discovered thus far do not belong to the PD…D/ExK family of enzymes, but rather have active sites typical of other endonuclease families. The present review deals with new developments in the field of Type II restriction endonucleases. One of the more interesting aspects is the increasing awareness of the diversity of Type II restriction enzymes. Nevertheless, structural studies summarized herein deal with the more common subtypes. A major emphasis of this review will be on target site location and the mechanism of catalysis, two problems currently being addressed in the literature.

Key words. Protein-nucleic acid interaction; facilitated diffusion; DNA recognition; DNA cleavage; mechanism of phosphodiester bond hydrolysis; evolution; protein engineering.

Introduction

Restriction endonucleases are components of restriction modification (RM) systems that occur ubiquitously among bacteria, archaea [1, 2] and in viruses of certain unicellular algae [3]. Their main function is to defend their host against foreign DNA. This is achieved by cleaving incoming DNA that is recognized as foreign by the absence of a characteristic modification (N4 or C5 methylation at cytosine or N6 methylation at adenine) at defined sites within the recognition sequence. The host DNA is resistant to cleavage as these sites are modified. Additional functions have been attributed to restriction enzymes, including maintenance of species identity among bacteria [4] and generation of genetic variation [5, 6]. Restriction endonucleases of *Chlorella* viruses may have a nutritive function by helping degrade host DNA or preventing infection of a cell by another virus [3]. Certain types of RM systems can also be considered as selfish DNA elements [7, 8]. In general, bacteria and archaea harbour numerous RM systems. For example, in *Helicobacter pylori* more than 20 putative RM systems, comprising greater than 4% of the total genome, have been identified in two completely sequenced *H. pylori* strains [9]. Several types of restriction endonucleases exist that differ in subunit composition and cofactor requirement. Commonly, four types are distinguished [10].

Type I restriction enzymes consist of three different subunits, HsdM, HsdR and HsdS, that are responsible for modification, restriction and sequence recognition,

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respectively. The quaternary structure of the active Type I restriction enzyme is $HsdM_2HsdR_2HsdS$. Type I enzymes require ATP, $Mg^{2+}$and AdoMet for activity. They interact in general with two asymmetrical bi-partite recognition sites, translocate the DNA in an ATP-hydrolysis dependent manner and cut the DNA distal to the recognition sites, approximately half-way between two sites. Typical examples are EcoKI, EcoAI, EcoR124I and StySBLI, which represent Type IA, IB, IC and ID subtypes, respectively [11–14].

Type III restriction enzymes consist of two subunits only, Mod (responsible for DNA recognition and modification) and Res (responsible for DNA cleavage). Active nucleases have a Mod_2Res_2 stoichiometry, require ATP and Mg^{2+} for activity and are stimulated by AdoMet. They interact with two head-to-head arranged asymmetrical recognition sites, translocate the DNA in an ATP-hydrolysis dependent manner and cut the DNA close to one recognition site. Typical examples are EcoP1I and EcoP15I [12–14].

Type IV restriction enzymes recognize and cleave methylated DNA. As such they are not part of an RM system. The best-studied representative is McrBC, which consists of two different subunits, McrB and McrC, responsible for DNA recognition and cleavage, respectively. McrBC recognizes DNA with at least two RC sequences at a variable distance, containing methylated or hydroxymethylated cytosine in one or both strands. For DNA cleavage GTP and Mg^{2+} are required; cleavage occurs close to one of the two RmC sites [12–14].

This review will deal with Type II restriction endonucleases with a particular focus on the structure and mechanism of these enzymes. For previous reviews see references [15–20].

Diversity of Type II restriction endonucleases

As of 29 October 2004, REBASE (http://rebase.neb.com/rebase/rebase.html) lists 3707 restriction enzymes: 59 Type I, 3635 Type II, 10 Type III and 3 Type IV. The predominance of Type II enzymes certainly is biased by their usefulness for recombinant DNA work. The analysis of published genome sequences suggests a somewhat more even distribution among putative RM systems: approximately 29% Type I, 45% Type II, 8% Type III and 18% Type IV [R. Roberts, personal comm.].

Type II restriction endonucleases differ from the Type I, III and IV enzymes by a more simplified subunit organization. They are usually homodimeric or homotetrameric enzymes that cleave DNA within or close to their recognition site and do not require ATP or GTP. With only one exception known to date (see below), they require Mg^{2+} as cofactor.

Orthodox Type II enzymes are homodimers that recognizes palindromic sequences of 4–8 bp in length, and cleave DNA within this sequence in both strands, producing 3′-hydroxyls and 5′-phosphate ends. Some recognize discontinuous palindromes, interrupted by a segment of specified length but unspecified sequence. The DNA fragments produced have ‘blunt’ or ‘sticky’ ends with 3′- or 5′-overhangs of up to 5 nucleotides (there is a single known example of an enzyme producing a 7-nucleotide 3′-overhang: TspRI (CASTGNN/)). Most of the restriction enzymes used for recombinant DNA work [21–23] belong to this subtype, which is called Type IIP (P for palindromic) according to the accepted nomenclature [10]. Many Type II restriction endonucleases have properties different from the Type IIP enzymes, for which EcoRI (recognition sequence G/AATTC) and EcoRV (GAT/ATC) are the best-known and best-studied representatives. The current nomenclature tries to group the Type II restriction enzymes according to properties that are unique to the respective subtype. However, as will be seen, overlap cannot be avoided. This is the consequence of the great diversity among Type II restriction endonucleases.

Type IIA enzymes recognize asymmetric sequences. An interesting member of this subtype is Bpu10I [CCTNAGC(-5/-2)], a dimer of non-identical subunits, each of which is responsible for cleavage of one strand of the DNA: 5′-CC/TNAGC-3′ and 5′-GC/TNAGG-3′ [24]. These enzymes are ideal precursors for the generation of nicking enzymes.

Type IIB enzymes cleave DNA at both sides of the recognition sequence, an example being BplI [(8/13)GAGNNNNNCTC(13/8)]. BplI cleaves the top strand 8 nucleotides before and 13 nucleotides after the recognition sequence, while the bottom strand is cleaved 13 nucleotides before and 8 nucleotides after the recognition sequence [25].

Type IIC enzymes have both cleavage and modification domains within one polypeptide. One of the first discovered was BcgI [(10/12)CGANNNNNNTGC(12/10)], which has a very unusual functional organization: it has an A_2B quaternary structure [26] with both endonuclease and methyltransferase domains in the A subunit and the target recognition domain located in the B subunit [27]. BcgI illustrates the problem of the nomenclature of Type II restriction endonucleases, as it is also a Type IIB enzyme.

Type IIE enzymes need to interact with two copies of their recognition sequence for efficient cleavage, one copy being the target for cleavage, the other serving as an allosteric effector [28–30]. The best-studied examples with respect to structure and function are EcoRII (/CCWGG) [31-36] and NaeI (GCC/CGG) [37-41]. It is interesting to note that the removal of the effector domain of EcoRII converts this Type IIE enzyme into a very active Type IIP enzyme [34]. Sau3AI (/GATC), in the absence of DNA a monomer with two similar domains,

dimerizes in the presence of DNA and then functions as a Type IIE enzyme, with a catalytic site and an allosteric effector site [42].

Type IIF enzymes are typically homotetrameric restriction endonucleases that also interact with two copies of their recognition site, but cleave both of them in a more or less concerted manner [28, 30, 43, 44]. Well-studied examples are Cfr10I (R/CCGGY) [45-47], NgoMIV (G/CCGGC) [47, 48] and SfiI (GGCCNNNN/NGGCC) [49, 50]. SgrAI (CR/CCGGYG), although a dimer in solution, assembles into a functional tetramer upon DNA binding [51, 52].

Type IIG enzymes, similar to and essentially a subgroup of Type IIC enzymes, have both cleavage and modification domains within one polypeptide. They are in general stimulated by AdoMet, but otherwise behave as typical Type II enzymes, though most are also Type IIS enzymes (see below). A well-studied example is Eco57I [CTGAAG (16/14)] [53, 54]. Type IIG enzymes are very promising for the engineering of restriction endonucleases with new specificities, as shown first for Eco57I [55].

Type IIH enzymes behave like Type II enzymes, but their genetic organization resembles Type I RM systems. AhdI, for example, recognizes the sequence GACNNN/NNGTC, but its companion methyltransferase consists of two modification and two specificity subunits [56].

Type IIM enzymes recognize a specific methylated sequence and cleave the DNA at a fixed site. The best-known representative is DpnI (GA/TC), which cleaves $G^{m6}ATC$, $G^{m6}AT^{m4}C$ and $G^{m6}AT^{m5}C$, yet not GATC, $GAT^{m4}C$, $GAT^{m5}C$ or certain hemimethylated sites. Note the difference between Type IIM and Type IV enzymes (such as McrBC), which do not cleave DNA at a fixed site. Many restriction enzymes are more or less tolerant to methylation (see, for example, [57]). For Type IIM enzymes the methyl group is an essential recognition element.

Type IIS enzymes cleave at least one strand of the target DNA outside of the recognition sequence [28, 30, 58]. One of the best-known Type IIS enzymes is FokI (GGATG(9/13) [59], which like many other Type IIS enzymes [60, 61] interacts with two recognition sites before cleaving DNA. Type IIS enzymes are active as homodimers and, from what is currently known, are composed of two domains, one responsible for target recognition and the other for catalysis (also serving as the dimerization domain). This is apparent from the crystal structure of FokI [62] and from biochemical studies of BfiI [63]. Type IIS enzymes have been used for the creation of rare restriction sites ('Achilles' heel cleavage [64]) and more recently for the generation of chimeric nucleases [65, 66] as well as strand-specific nicking endonucleases [67].

Type IIT enzymes are heterodimeric enzymes. A recently characterized representative is BslI (CCNNNNN/NNGG), which is composed of two different subunits. The functional restriction endonuclease presumably is a $\alpha_2\beta_2$ tetramer [68]. Several of these enzymes have been used to generate nicking enzymes, viz. BbvCI, BsaI, BsmAI, BsmBI and BsrDI [69].

Some Type II restriction enzymes only nick DNA**.** DNA nicking endonucleases were found in *Bacillus stearothermophilus*, for example Nt.BstNBI and Nt.BstSEI ($GAGTCN_4$/) [70, 71], and in *Chlorella* viruses, for example Nt.CviPII (/CCD) and Nt.CviQXI (R/AG) [72, 73]. Nt stands for Nicking enzyme with top strand cleavage activity. These nicking enzymes are very useful for the isothermal amplification of DNA.

Many Type II restriction enzymes have not yet been characterized in detail, and for quite a number of enzymes it is not known whether they belong to Type IIP, E or F. Quaternary structure analysis of the active enzyme and testing against substrates with one or two copies of the recognition sequence are required to determine if the enzyme needs to bind to two sites and, if so, how many phosphodiester bonds are cleaved per turnover. Such a study was carried out for seven Type II restriction enzymes that all recognize the same sequence (GGCGCC) but cleave it at four different positions, for example isoschizomers (cleavage at the same position) and neoschizomers (cleavage at different positions): BbeI, EgeI, Mly113I, EheI, KasI, NarI and SfoI. SfoI, EgeI and EheI were found to be Type IIP enzymes, Mly113I and BbeI are Type IIF enzymes (with mechanistic peculiarities), NarI can be considered an unorthodox Type IIE enzyme with a preferential nicking activity and its neoschizomer KasI appears to be an unorthodox Type IIP enzyme with a preferential nicking activity. It was concluded from this study that the range of cleavage modes is larger than typically imagined, as is the number of enzymes needing two recognition sites [74].

Three dimensional structures of Type II restriction endonucleases

Crystal structure information is available for 16 Type II restriction endonucleases of the PD…D/ExK superfamily and seven other nucleases belonging to this family (table 1). Figure 1A shows 9 structures of free restriction endonucleases, and figure 1B shows 13 structures of specific enzyme-DNA complexes. A comparison of these crystal and co-crystal structures illustrates that these enzymes have a similar core which harbours the active site (one per subunit) and which serves as an important structure stabilization factor ('stabilization centre') [75]. This core comprises a five-stranded mixed β-sheet flanked by α-helices [76]; the second and third strand of the β-sheet serve as a scaffold for the catalytic residues of the PD…D/ExK motif. The fifth β-strand can be parallel

Table 1. Crystal structures of Type II restriction endonucleases of the PD…D/ExK family and related enzymes.

Enzyme	Subtype	Recognition site	Catalytic residues	PDB code (reference)
EcoRI-like				
BamHI	IIP	G$^{\downarrow}$CTAGG	Asp94;Glu111;Glu113	1BAM (apo) [146], 1BHM (+ spec. DNA) [211], 2BAM (+ spec. DNA, Ca^{2+}), 3BAM (product, Mn^{2+}) [154], 1ESG (+ non-spec. DNA) [212]
BglII	IIP	A$^{\downarrow}$GATCT	Asp84;Glu93;Gln95	1ES8 (apo) [213], 1DFM (+ spec. DNA, Ca^{2+}), 1D2I (+ spec. DNA, Mg^{2+}) [147]
BsoBI	IIP	C$^{\downarrow}$PyCGPuG	Asp212;Glu240;Lys242	1DC1 (+ spec. DNA) [134]
Bse634I	IIF	Pu$^{\downarrow}$CCGGPy	Asp146;Glu212;Lys198	1KNV (apo) [192]
Cfr10I	IIF	Pu$^{\downarrow}$CCGGPy	Asp134;Glu204;Lys190	1CFR (apo) [45]
EcoRI	IIP	G$^{\downarrow}$AATTC	Asp91;Glu111;Lys113	1ERI (+ spec. DNA) [214], 1QC9 (apo), 1QPS (product, Mn^{2+}), 1CL8 (+ mod. DNA), 1QRH (R145K) (+ spec. DNA), 1QRI (E144D) (+ spec. DNA)
EcoRII	IIE	$^{\downarrow}$CCWGG	Asp299;Glu337;Lys324	1NA6 [36]
FokI	IIS	GGATG$N_9^{\downarrow}$NNNN$^{\uparrow}$	Asp450;Asp467;Lys469	2FOK (apo) [62], 1FOK (+ spec. DNA) [78]
MunI	IIP	C$^{\downarrow}$AATTG	Asp83;Glu98;Lys100	1D02 (D83A) (+ spec. DNA) [141]
NgoMIV	IIF	G$^{\downarrow}$CCGGC	Asp140;Glu201;Lys187	1FIU (product, Mg^{2+}) [48]
Related to EcoRI				
TnsA (Tn7transposase)			Glu63;Asp114;Lys132	1F1Z (Mg^{2+}) [215]
EcoRV-like				
BglI	IIP	GCCNNNN$^{\downarrow}$NGGC	Asp116;Asp142;Lys144	1DMU (+ spec. DNA, Ca^{2+}) [155]
EcoRV	IIP	GAT$^{\downarrow}$ATC	Asp74;Asp90;Lys92	1RVE (apo) [130], 1AZ3, 1AZ4 (apo) [216], 4RVE (+ spec. DNA) [130], 1B95 (+ spec. DNA) [217], 1AZ0 (+ spec. DNA, Ca^{2+}) [216], 1B94 (+ spec. DNA, Ca^{2+}) [217], 1EOO (+ spec. DNA) [169],1RVA (prod, Mg^{2+}) [168], 1BSS (T93A) (+ spec. DNA, Ca^{2+}) [219],1B96 (Q69E) (+ spec. DNA), 1B97 (Q69L) (+ spec. DNA) [217], 1SUZ (K92A) (+ spec. DNA, Mg^{2+}), 1SX8 (K92A) (+ DNA, Mn^{2+}), 1STX, 1SX5 (K38A) (product, Mn^{2+}), [159], 1BSU, 1BUA (+ mod. DNA) [218], 1RV5 (+ interrupted DNA) [220], 1EO3, 1EON (+ mod. DNA) [169], 2RVE (+ non-spec. DNA) [130],1RVB (+ non-spec. DNA, Mg^{2+}) [168]
HincII	IIP	GTPy$^{\downarrow}$PuAC	Asp114;Asp127;Lys129	1KC6 (+ spec. DNA) [117] 1TW8 (+ spec. DNA, Ca^{2+}) [157], 1HXV (prod, Mg^{2+}) [158], 1XHU (product Mn^{2+}) [158],
MspI	IIP	C$^{\downarrow}$CGG	Asp99;Asn117;Lys119	1SA3 (+ spec. DNA) [79]
NaeI	IIE	GCC$^{\downarrow}$GGC	Asp86;Asp95;Lys97	1EV7 (apo) [39], 1IAW (+ spec. DNA) [40]
PvuII	IIP	CAG$^{\downarrow}$CTG	Asp58;Glu68;Lys70	1PVU (apo) [221], 1K0Z (apo) (Pr^{3+}), 1H56 (apo) (Mg^{2+}) [222], 1PVI (+ spec. DNA) [223], 1EYU (+ spec. DNA, pH 4.6) [156], 1F0O (+ spec. DNA, Ca^{2+}, crosslinked, pH 7.5) [156], 2PVI (+ mod. DNA) [224], 3PVI (D34G) (+ spec. DNA [219], 1NI0 (Y94F)
Related to EcoRV				
λ-exonuclease		non-specific	Asp119;Glu129;Lys131	1AVQ [225]
RecB endonuclease		non-specific	Asp1067;Asp1080;Lys1082	1W36 [226]
S.solfataricus Hjc resolvase		structure specific	Asp42;Glu55;Lys57	1HH1 [227]
MutH		$^{\downarrow}$GATC	Asp70;Glu77;Lys79	1AZO, 2AZO [81]
T7 endonuclease I		structure specific	Asp55;Glu65;Lys67	1FZR (E65K) [228] 1M0I [229] 1M0D (Mn^{2+}) [229]
VSR endonuclease		C$^{\downarrow}$TWGG	Asp51	1VSR [82] 1CW0 [83] 1OGD [230]

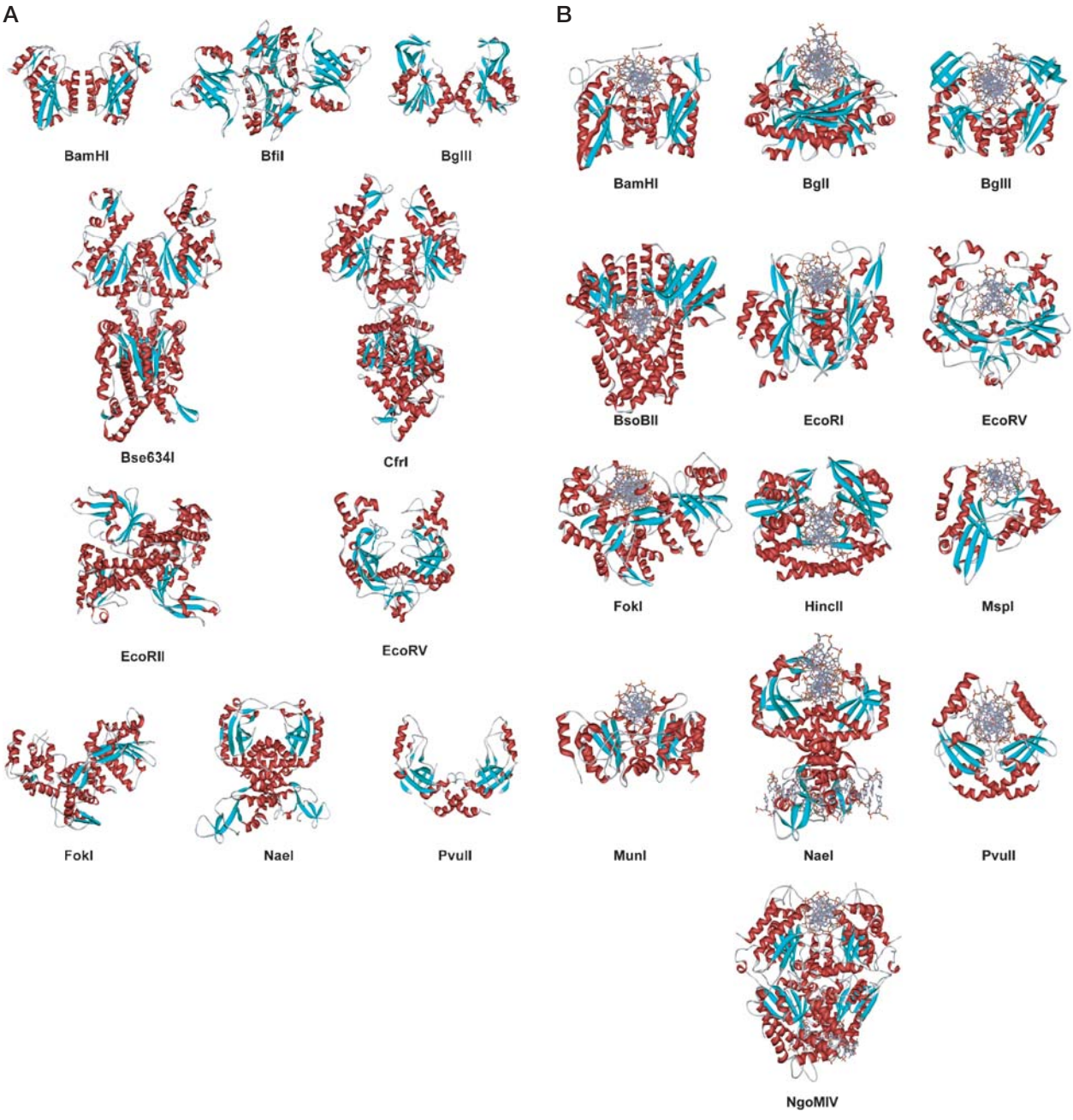

Figure 1. Crystal structures of Type II restriction endonucleases. (*A*) free enzymes: BamHI (1BAM), BfiI [V. Siksnys, unpublished], BglII (1ES8), Bse634I (1KNV), Cfr10I (1CFR), EcoRII (1NA6), EcoRV (1RVE), FokI (2FOK), NaeI (11EV7), PvuII (1PVU); (*B*) specific restriction endonuclease – DNA complexes: BamHI (2BAM), BglI (1DMU), BglII (1DFM), BsoBI (1DC1), EcoRI (1ERI), EcoRV (4RVE), FokI (1FOK), HincII (1KC1), MspI (1SA3), MunI (1DO2), NaeI (1IAW), PvuII (1PVI), NgoMIV (1FIU). α-helices are indicated in red, β-strands in blue. Note that Bse634I, Cfr10I and NgoMIV are homotetrameric enzymes, EcoRII and NaeI as Type IIE enzymes have an extra domain and FokI and MspI are monomeric enzymes in the co-crystal.

(as in EcoRI) or antiparallel (as in EcoRV) to the fourth strand [39] (fig. 2). Based on structural differences, in particular the topology of secondary structure elements and the arrangement of the subunits, the enzymes of the PD…D/ExK superfamily of Type II restriction endonucleases can be divided into an EcoRI and EcoRV branch [39, 77]. Enzymes that belong to the EcoRI branch (BamHI, BglII, Bse634I, BsoBI, Cfr10I, EcoRI, EcoRII, FokI, MunI, NgoMIV) usually approach the DNA from the major groove [77], recognize the DNA mainly via an α-helix and a loop (α-class [39]) and in general produce 5′-staggered ends. Enzymes of the EcoRV branch (BglI, EcoRV, HincII, NaeI, MspI, PvuII) usually approach the DNA from the minor groove [77], use a β-strand and a β-

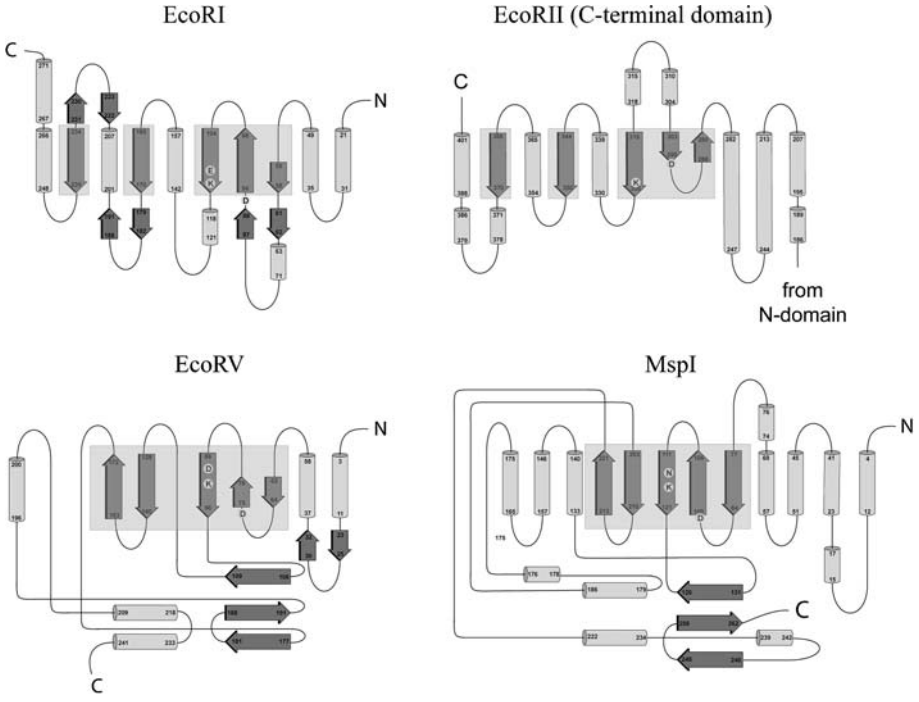

Figure 2. Topologies of the Type II restriction endonucleases EcoRI, EcoRII (catalytic domain), EcoRV and MspI. EcoRI and EcoRII belong to the α-family (EcoRI family), EcoRV and MspI to the β-family (EcoRV family); both have a central five-stranded β-sheet. The amino acid residues of the PD…D/ExK motif are located on the second and third strand, of this β-sheet. Whereas in the α-family the fifth strand is parallel to the fourth strand it is antiparallel in the β-family. The location of the PD…D/ExK motif is indicated in the topology diagram. α-helices are shown in light grey, β-strands in dark grey. The central five-stranded β-sheet is shaded grey.

like turn for DNA recognition (β-class [39]) and in general produce blunt or 3′-staggered ends. Both FokI [62, 78] and MspI [79] are monomers in the crystal. While FokI is known to dimerize on the DNA [80], the quaternary structure of MspI in its functional state is not yet known; however, analytical ultracentrifuge experiments show that in solution it exists in monomer-dimer equilibrium. Two nucleases of the PD…D/ExK superfamily, MutH and Vsr, are monomers in the crystal [81–83] and presumably are active as monomers, since both cleave only one strand of their DNA substrate.

Orthodox Type II restriction endonucleases are dimers of identical subunits that are composed of a single domain (with subdomains being responsible for DNA recognition, DNA cleavage and dimerization). The Type IIE enzymes (EcoRII [36] and NaeI [39, 40]) and the Type IIS enzymes (FokI [62, 78]) have a two-domain organization. These enzymes have a catalytic domain typical of the PD…D/ExK superfamily and a DNA binding domain that, in the case of the Type IIE enzymes, serves as an effector domain, and in the case of the Type IIS enzymes is responsible for DNA-recognition. The DNA-recognition domain of FokI and the effector domain of NaeI resemble the helix-turn-helix-containing DNA binding domain of the catabolite gene activator protein (CAP) [39, 78]. Whereas it was originally believed that the effector domain of EcoRII has a novel DNA recognition fold [36], comparison with the structure of the Type IIS enzyme BfiI (see below) shows that it rather resembles the DNA-recognition domain of BfiI [V. Siksnys, personal comm.].

For a long time it seemed as if all Type II restriction endonucleases belonged to the PD…D/ExK superfamily. The first enzyme that was unequivocally demonstrated not to be a member of this family was BfiI, a Type IIS enzyme [84] that does not require Mg^{2+} for DNA cleavage. Based on sequence similarity with a non-specific nuclease from *Salmonella typhimurium*, BfiI is considered to be a member of the phospholipase D superfamily [85]. BfiI is a homodimer with one catalytic centre [86] which is used to cut two DNA strands within one binding event [87]. The crystal structure of BfiI is shown in figure 1A.

There is bioinformatic evidence that a few other restriction enzymes do not belong to the PD…D/ExK superfamily, but rather are related to the H-N-H and GIY-YIG families of homing endonucleases [88–90]. KpnI, which has an H-N-H sequence motif [91], unlike all other restriction enzymes known is active in the presence of Ca^{2+}, whereas in the presence of Mg^{2+} or Mn^{2+} it shows a degenerate specificity [92]. It is noteworthy that the H-N-H homing endonuclease I-CmoeI is also active with Ca^{2+} [93].

Target site location

To bind to DNA restriction enzymes have to 'open' their DNA binding site. In several cases, however, structural information of the free enzyme implies that the DNA binding site does not appear to be sufficiently open to allow DNA binding. Whereas in most instances the enzymes make their binding sites accessible in a 'tong-like' motion, which is perpendicular to the DNA axis (*e.g.* BamHI, EcoRV, PvuII), BglII uses a 'scissor-like' motion, which is parallel to the DNA axis [94]. The question arises whether restriction enzymes oscillate between closed and open states, or whether the open state is induced by association of enzyme with DNA. For EcoRV it was demonstrated that there is an 'external binding' site, which when occupied by DNA may open the gate of the 'inner' DNA binding site [95].

All restriction endonucleases face the problem of efficiently finding their specific site in the presence of a huge excess of non-specific sites, to which they can also bind although with a much lower affinity [96]. EcoRI was the first restriction enzyme for which evidence was presented that it makes use of facilitated diffusion for target site location [97–99]. Facilitated diffusion is a very effective process that not only speeds up target site location by a factor > 10 [98], but also increases the processivity of restriction endonucleases [99] and accelerates the dissociation from the specific site after cleavage [97]. Under optimum conditions restriction endonucleases can scan ~10^6 bp in one binding event; due to the random movement on the DNA, the effective distance scanned is ~1000 bp [98].

Three principally different, but not mutually exclusive, mechanisms can account for the efficiency of target site location by DNA-binding proteins: (i) 'sliding'; (ii) 'jumping' or 'hopping'; and (iii) intersegment transfer [100–104].

Sliding (also called linear or one-dimensional diffusion) implies that the protein stays bound to the DNA after the first encounter and moves along the DNA by a random movement, following the pitch of the double helix until it finds its specific site or dissociates [105, 106]. This means that specific sites tend to not be overlooked by a restriction enzyme sliding along the DNA. During linear diffusion the non-specific binding mode is not given up and the water layer around DNA and protein, characteristic for the non-specific binding mode, remains largely intact (the number of water molecules sequestered in non-specific complexes is not known; for EcoRI it was estimated that in the transition from non-specific to specific complex ~100 water molecules are released at the protein-DNA interface [107]). Small ligands binding to the major (e.g. triple-helix forming oligodeoxynucleotides) or minor groove (distamycin, netropsin) of DNA are likely to pose a major obstacle for sliding. An alternative model has been proposed for sliding, which envisages movement on the surface of the DNA [108].

Jumping or hopping is normal (three-dimensional) diffusion that takes into account that the chance of reassociation of a DNA binding protein to the DNA molecule close to the site it has dissociated from is much greater than associating with another DNA molecule or a distant site on the same molecule. During jumping or hopping the non-specific binding mode is given up and the water layer characteristic for the free DNA and the free protein is re-formed. Jumping and hopping does not follow the pitch of the double helix, meaning that specific sites along the DNA can be overlooked during hopping, depending on the step size. Small ligands binding to DNA, such as intercalating drugs, minor groove binders or triple-helix forming oligodeoxynucleotides, should not pose a major obstacle for jumping or hopping.

Intersegment transfer is only possible for proteins that have two DNA binding sites. If the DNA is released from one binding site, the enzyme still remains bound to the DNA with the other binding site and can bind to the same DNA molecule at a distant location via its free DNA binding site. Binding of DNA to both DNA binding sites will produce loops in the DNA. Intersegment transfer is a particularly efficient way of covering large distances exceeding the persistence length of DNA. Due to steric constraints intersegment transfer is not an effective means for covering small distances in search of a specific site. Intersegment transfer is unlikely to be inhibited by small or even large DNA binding ligands.

The efficiency of facilitated diffusion can be easily tested by measuring the dependence of the rate of cleavage of a single-site substrate on the length of the DNA under conditions where target site location is limiting for cleavage [98, 105, 109]. Alternatively, one can measure the processivity of a restriction endonuclease in cleaving a second site on the same DNA molecule [99, 110–113]. It must be pointed out that there is an important principal difference between studies measuring directly the rate of target site location and those measuring the degree of processivity (which are influenced by the rate of dissociation from the target site after cleavage). The results obtained with these two types of measurements may but need not correlate.

There is agreement based on such experiments that restriction enzymes use facilitated diffusion to locate their target site. It is an open question, however, what the relative contributions of sliding and hopping are. Presumably, both one- and three-dimensional pathways are involved [102, 114], yet to what extent depends on the conditions, in particular Mg^{2+} concentration and ionic strength [99, 109], but also on the structure of the DNA binding site of the restriction enzyme. PvuII, EcoRV and BsoBI, which have an open, half-closed and fully closed DNA binding site (see also fig. 1A), respectively, make

use of facilitated diffusion to different extents: BsoBI > EcoRV >> PvuII [M. Specht, W. Wende and A. Pingoud, unpublished]. The importance of sliding can be deduced from the finding that triple-helix formation over 16 bp interferes with efficient target site location [105] and from the fact that an EcoRV molecule whose DNA binding site has been closed by a cross-link after non-specific binding to a circular DNA molecule is perfectly able to find and cleave its specific site after addition of Mg^{2+} [95]. In addition, during linear diffusion specific sites are not overlooked [105, 109, 115], which is most easily explained by sliding as a major determinant for facilitated diffusion. On the other hand, experiments with catenated circular DNA molecules demonstrate that jumping and hopping are also of importance for efficient target site location [111].

Non-specific (NS1) as well as specific DNA binding proteins (Myb, serum response factor) were shown to interfere with facilitated diffusion [98, 105]. This raised the question whether facilitated diffusion is of importance in vivo. Experiments in which phage restriction by EcoRV variants that differed in their ability to slide along the DNA was determined clearly showed that EcoRV makes use of facilitated diffusion in vivo [116].

For two enzymes, BamHI and EcoRV, structural information is available for the free enzyme, the non-specific enzyme-DNA complex (for BamHI the non-specific complex is a complex with an oligodeoxynucleotide with a sequence differing in one base pair from the recognition sequence; for EcoRV the non-specific complex is a complex with two stacked octadeoxynucleotides), the specific enzyme-DNA complex and an enzyme-product complex (fig. 3). The non-specific complex presumably closely reflects the structure of the complex that slides along the DNA, whereas the specific complex crystallized in the presence of Ca^{2+} can be considered as resembling the enzyme-substrate complex. Our understanding of the mechanism of DNA recognition by Type II restriction endonucleases is mainly due to the structure analyses of such complexes, with additional thermodynamic and kinetic analyses employing enzyme variants (e.g. Horton, Otey et al. [117]) and chemically modified substrates (e.g. Kurpiewski, Engler et al. [118]).

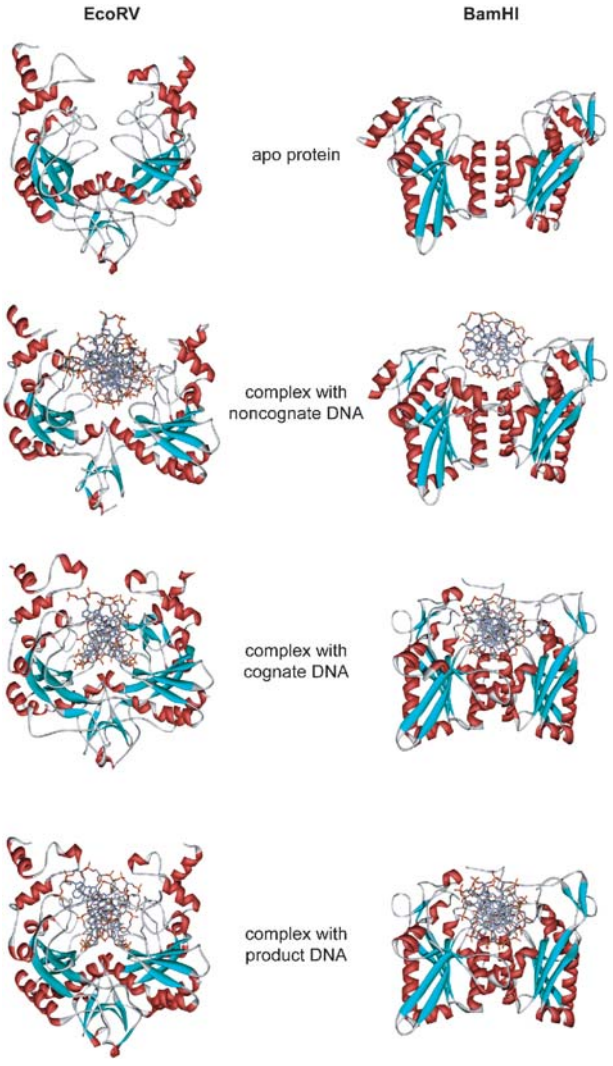

Figure 3. Comparison of the crystal structures of the free enzyme (top), the non-specific complex, the enzyme-substrate and the enzyme-product complex (bottom) for the Type II restriction endonucleases EcoRV (left) and BamHI (right).

Recognition

The recognition process in general consists of conformational adaptations of protein and DNA with water and counter-ion release at the protein-DNA interface [119]. This results in a favourable ΔH contribution from direct protein-DNA recognition interactions and a favourable ΔS contribution from water and counter-ion release, likely to compensate the unfavourable ΔS contribution due to the immobilization of amino acid side chains at the protein-DNA interface [120]. Major distortions of the DNA will decrease the favourable ΔH contribution because of base-pair destacking. Still, in several instances specific complex formation of restriction endonucleases with their DNA substrate is associated with kinking or bending of the DNA (e.g. EcoRI [121]; EcoRV [122]). The ease with which a DNA sequence can be distorted at a defined site upon interaction with a restriction endonuclease can be used for the recognition process. For example, the central TpA step in the EcoRV recognition sequence is sharply bent upon specific interaction with EcoRV; a TpA step is more flexible and easier to unstack than other dinucleotide steps [123]. This may explain why EcoRV needs only a single hydrophobic contact per subunit (between the methyl groups of Thr186 and the

central thymine of the recognition sequence GATATC) to recognize the central two base pairs. Introducing a distortion in the free DNA, which is normally only obtained in the presence of the enzyme, can lead to very powerful inhibitors [124]. Interestingly, some restriction endonucleases require the presence of the divalent metal ion cofactor Mg^{2+} (or its analogue Ca^{2+}) for specific binding, as first shown for EcoRV [125, 126]. For MunI, it was demonstrated that the Ca^{2+} dependence of specific binding is relieved at low pH or by replacing the active site carboxylates with alanine, suggesting that the unprotonated carboxylates at the active site repel the DNA and prevent specific complex formation [127, 128]. This cannot be the complete explanation for EcoRV, because replacing the active site carboxylates by alanine does not relieve the Mg^{2+} dependence for specific binding [129] but rather suggests that Mg^{2+} binding sites distant from the active site must exist and are required for specific binding [17]. The structure of the specific EcoRV-DNA complex crystallized in the absence of divalent metal ions had all the features of a recognition complex [130], including the pronounced bending of the DNA by ~50°, as also determined in solution for an inactive EcoRV variant in the presence of Mg^{2+} [131]. A recent FRET study, however, demonstrated that in solution EcoRV does not bend its DNA substrate in the absence of divalent metal ions [132], suggesting that in the case of EcoRV crystal packing forces favoured a high-energy complex resembling the recognition complex that in solution is not populated in the absence of divalent metal ions. It would be interesting to know whether a similar result would be obtained for HincII, which in the crystal bends the DNA by ~45° in the absence of divalent metal ions [133].

Inspection of the available co-crystal structures of specific restriction enzyme-DNA complexes allows the following generalizations to be made regarding the structural aspects of the recognition process:

1) Most enzymes that produce blunt ends (such as EcoRV) or sticky ends with 3′-overhangs (such as BglI) approach the DNA from the minor groove (HincII ([133] is the only exception so far!), whereas enzymes that produce sticky ends with 5′-overhangs (such as EcoRI) contact the DNA from the major groove.

2) Specific DNA-binding is accompanied by more or less pronounced distortions of the DNA that bring functional groups of the DNA into positions required for optimal recognition, but also position the scissile phosphates vis à vis the catalytic centre and the 3′-proximal phosphates such that they can support phosphodiester bond hydrolysis. Specific DNA binding is accompanied by conformational changes in the protein that involve structuring regions that are unstructured in the free enzyme or in the non-specific complex [in BamHI the C-terminal nine amino acid residues, which are α-helical in both the free enzyme and the non-specific complex, unfold and make contacts to the minor groove (R-subunit) and the sugar-phosphate backbone (L-subunit), respectively]. These conformational changes often involve a repositioning of the subunits and of the subdomains. In the specific complex the DNA is partially (e.g. PvuII: 157 amino acid residues/subunit) or as in most cases fully (e.g. BsoBI: 323 amino acid residues/subunit) encircled by the restriction endonuclease. BsoBI is an extreme case as it forms a tunnel around its DNA substrate [134]. Unique among restriction endonuclease-DNA complexes is the intercalation of amino acid residues into the DNA double helix, as observed for HincII, where glutamine side chains (one from each subunit) penetrate the DNA on either side of the recognition site [133].

3) The formation of a highly co-operative hydrogen bond network is a characteristic feature of the specific protein-DNA complex of restriction endonucleases. This hydrogen bond network comprises contacts to the bases ('direct read-out') as well as to the sugar-phosphate backbone ('indirect readout'). A majority of the possible hydrogen bonds, mostly direct but also water-mediated, are formed to the edges of the bases in the major groove (e.g. BamHI: 14/18; BglII: 12/18 [94]) and often in the minor groove (e.g. BamHI: 6/12; BglII: 10/12 [94]), most of them are direct, but a few are water-mediated. In addition, van der Waals interactions and hydrophobic contacts are formed to the bases of the recognition sequence. The phosphates of the backbone of the recognition sequence are engaged in Coulombic interactions (e.g. BamHI: 8; BglII: 6 [94]) and in many mostly water-mediated hydrogen bonds (e.g. BamHI: 28; BglII: 36 [94]). In general, several non-contiguous chain segments of a restriction enzyme are involved in direct and indirect readout. Whereas most of the specific contacts are between one subunit and one half-site of the palindromic recognition sequence, a few are directed to the other half-site. A characteristic feature of the recognition process is its high redundancy; this perhaps is the major reason why efforts to change the specificity of Type II restriction endonucleases by rational protein design by and large have been unsuccessful [135, 136].

Coupling between recognition and catalysis

Coupling between recognition and catalysis is one of the least-understood aspects of the enzymology of Type II restriction endonucleases. What one would like to know is how residues involved in direct and indirect readout communicate with the catalytic centres and trigger conformational changes that are required for the initiation of phosphodiester bond cleavage. Crystal structure analyses together with molecular dynamics simulations on one side and detailed thermodynamic and kinetic studies on the other side are required to supply the information needed to develop a chemically and structurally satisfac-

tory model of the path from the ground state to the transition state. However, crystal structures can be misleading, especially when they represent inactive complexes. This was the case with EcoRV, where until recently only inactive complexes with the divalent metal ion cofactor(s) in non-productive position could be crystallized [122].

Coupling between recognition and catalysis also means co-ordination of the two catalytic centres. It has been established for most Type II restriction enzymes that they cleave the two strands of their double-stranded substrate in a concerted manner. Insight into this coupling was provided by heterodimer experiments with EcoRV, in which amino acid residues involved in recognition or catalysis were substituted only in one subunit [137]. These experiments clearly showed that the substitution of an amino acid residue responsible for base recognition in one half-site of the palindromic recognition sequence dramatically reduces cleavage activity of the heterodimeric variant. This means that there is a cross-talk between amino acid residues involved in a base-specific contact in one subunit with the catalytic centres of both subunits. This guarantees that DNA cleavage is only initiated when all base specific contacts have been made. It is very likely that in EcoRV this crosstalk is mediated by the interaction between the tips of the two recognition loops [130]. In contrast, substitution of an amino acid residue of the catalytic centre did not affect the activity of the catalytic centre of the other subunit; neither did amino acid substitutions of several residues involved in indirect readout [138, 139]. Similar results were obtained for PvuII using a single-chain variant [140]. Intersubunit crosstalk in EcoRI is mediated by Glu144 and Arg145 [121]. These residues are part of the 137–145 segment that is responsible for most of the base-specific contacts in EcoRI. Arg145 in each subunit (A) is hydrogen bonded through its guanidino group to Glu144 of the other subunit (B) forming a ringlike structure involving the peptide backbones 144A–145A and 144B–145B and the side chains Arg145A–Glu144B and Arg145B–Glu144A ('crosstalk ring' [118]). A very similar arrangement is seen in the co-crystal structure of MunI with Glu120 and Arg121 [141].

Mechanism of phosphodiester bond hydrolysis

Phosphodiester bond hydrolysis by Type II restriction endonucleases follows an S_N2-type mechanism, which is characterized by inversion of configuration at phosphorous [142, 143]. The general mechanism of phosphodiester hydrolysis comprises three steps: (i) the preparation of the **attacking nucleophile** by deprotonation,

$$H_2O + B + R'\text{-}O5'\text{-}(PO_2)^-\text{-}O3'\text{-}R'' \rightarrow \mathbf{OH^-} + BH^+ + R'\text{-}O5'\text{-}(PO_2)^-\text{-}O3'\text{-}R''$$

(ii) the nucleophilic attack of the hydroxide ion on the phosphorous leading to the formation of the **pentavalent transition state**,

$$OH^- + R'\text{-}O5'\text{-}(PO_2)^-\text{-}O3'\text{-}R'' \rightarrow \mathbf{R'\text{-}O5'\text{-}(PO_2OH)^{2-}\text{-}O3'\text{-}R''}$$

(iii) the departure of the **3′ hydroxyl leaving group**:

$$-O5'\text{-}(PO_3)^{2-}\text{-}O3'\text{-} + H_2O \rightarrow R'\text{-}O5'\text{-}(PO_2)\text{-}O^-\text{-} + \mathbf{HO3'\text{-}R''} + OH^-$$

To achieve efficient catalysis, all three steps require an assisting group: (i) a base to deprotonate the water molecule; (ii) a Lewis acid that stabilizes the pentavalent transition state with two negative charges; and (iii) an acid that protonates the leaving 3′-oxyanion. The mechanism of restriction endonuclease catalysis can only be described if these groups are identified.

The catalytic centres of Type II restriction endonucleases generally contain a PD…D/ExK motif [77, 144, 145]. The negatively charged side chains serve to ligate a divalent metal ion cofactor, usually Mg^{2+} that is obligatory for catalysis. Lysine that is often considered as a general base candidate, however, is not strictly conserved; for example, in BamHI it is replaced by glutamate [146], and in BglII by glutamine [147]. The major controversy regarding the mechanism of DNA cleavage by restriction endonucleases is about the number of divalent metal ions involved in the catalytic process. This is attributable to some crystal structures having one divalent cation, while others have two divalent cations associated with the catalytic centre, or that the divalent metal ions are located in different positions, or that individual subunits in a crystal differ in divalent metal ion occupancy [17, 148]. The mechanistic models for DNA cleavage by restriction endonuclease are based on the number of metal ions involved in the reaction. Accordingly, three mechanisms with several variations were proposed (fig. 4).

One-metal ion mechanism

This mechanism requires a single metal ion at the active site that stabilizes the developing negative charge during the nucleophilic attack. The deprotonation of the attacking water molecule is accomplished by the phosphate group that is 3′ to the scissile bond. This mechanism is, therefore, often referred to as substrate-assisted [149]. It is supported by co-crystal structures with a single divalent metal ion at the active site (EcoRI [121], BglII [147]) and results of cleavage experiments with modified substrates, containing phosphorothioate or methylphosphonate substitutions at the phosphate 3′ to the scissile phosphate [118, 149–151], which lead to an almost complete loss of cleavage activity.

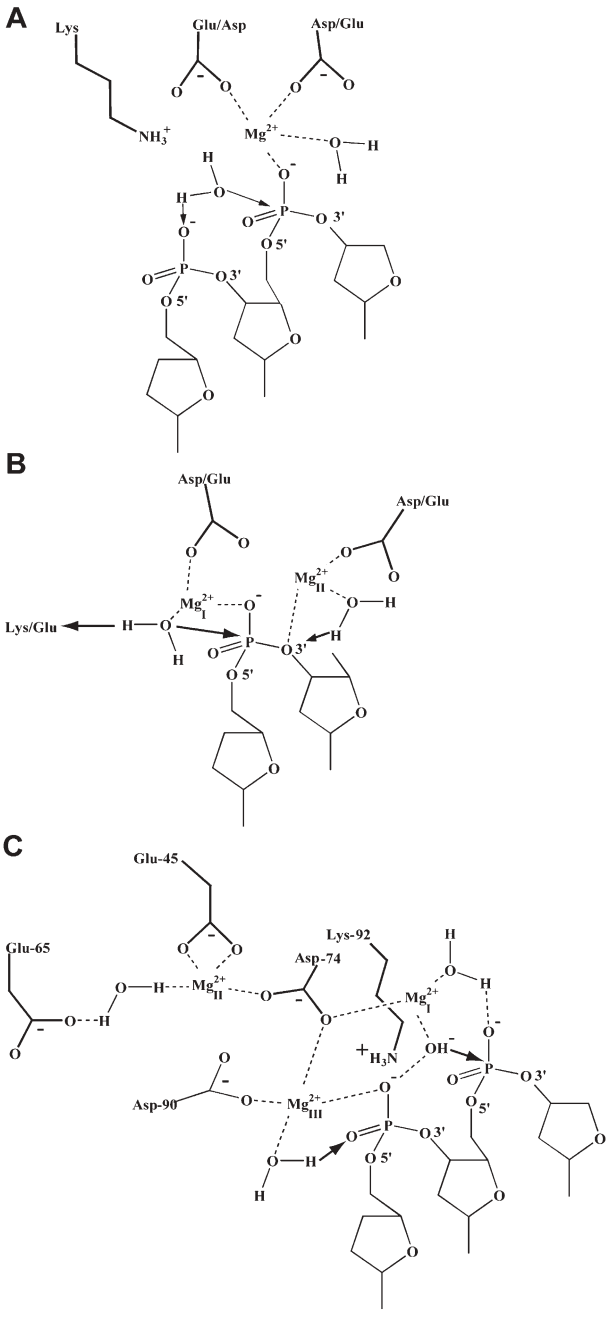

Figure 4. Three models proposed for the mechanism of phosphodiester bond cleavage by Type II restriction endonucleases. (*A*) *One-metal-ion mechanism.* One metal ion is bound at the active site and helps to lower the pK_a of the neighbouring water molecule and stabilizes the transition state of the nucleophilic attack. The nucleophile is generated from the precursor water molecule by the phosphate 3′ to the scissile phosphate. (*B*) *Two-metal-ion mechanism*. Two metal ions are located at the active site parallel to the apical direction of the pentavalent transition state. The OH^- is generated by a general base, lysine or glutamic acid. Both metal ions are important to reduce the electrostatic repulsion at the transition state, by ligating the incoming O and leaving O3′ atoms. (*C*) *Three-metal-ion mechanism*. Three positions are occupied by the two metal ions during the reaction, although only two of them are catalytically important. The metal ion in site I is bound to the attacking water molecule and facilitates its deprotonation, whereas metal ion III is dominant in transition state stabilization. Metal ion II has mostly a structural role.

Two-metal ion mechanism

This mechanism is adapted from the mechanism first put forward for the *Escherichia coli* DNA polymerase I 5′-3′exonuclease reaction [152, 153], where two divalent metal ions are used to accelerate the phosphodiester bond hydrolysis reaction. One of the metal ions is responsible for lowering the pK_a of a neighbouring water molecule, facilitating its deprotonation. Both metal ions are required to stabilize the doubly charged pentavalent transition state: they should lie in parallel with the apical direction of the trigonal bipyramide, one interacting with the oxygen of the nucleophile that forms the bond with the phosphorous, while the other interacts with the leaving 3′-oxyanion group. This ideal arrangement, when the metal ions are located ~4 Å from each other, is the most efficient to reduce the electrostatic repulsion between negative charges that accumulate at the transition state. This mechanism is supported by various co-crystal structures of pre-reactive and post-reactive complexes (BamHI [154], BglI [155], NgoMIV [48] and PvuII [156]). HincII is a problematic case; in the presence of Ca^{2+} only one metal ion is seen in the pre-reactive complex [157], whereas in the post-reactive complex two Mg^{2+} or Mn^{2+} ions are present [158]. The authors favour a two-metal ion mechanism.

Three-metal ion mechanism

Although no crystal structure with three metal ions bound is available, based on the different positions of the metal ions at the EcoRV active site a catalytic mechanism using two metal ions in three different positions has been proposed (Horton and Perona [159]). The metal ion in site I, which corresponds to site I or A in the two-metal ion mechanism, facilitates the formation of the nucleophilic hydroxide, whereas the metal ion in site III, which corresponds to site II or B in the two-metal ion mechanism, provides the major contribution to stabilisation of the transition state of the nucleophilic attack step. Initially metal site III is occupied, and this metal ion is shifted later to position II. This metal ion is also proposed to reduce the pK_a of a water molecule that protonates the leaving 3′-oxyanion.

The generalization of any of the three models for all restriction enzymes is hindered by conflicting experimental results. Direct involvement of the 3′ phosphate in the generation of the attacking nucleophile can be argued based on the high pK_a shift (~6 pH units) that is required for the phosphate to act as a general base [160], providing ~8 kcal/mol unfavourable contribution to the activation barrier of the reaction. Furthermore, substitution of phosphates that are 4–5 bp away from the cleavage site still affect catalysis, suggesting an electrostatic control rather than a direct involvement of these groups in catalysis [151]. The stringent geometric criteria for the arrangement of metal ions in the two-metal ion mechanism may

not be fulfilled in the active site of all restriction enzymes. In any case, EcoRI shows no evidence so far of binding a second metal ion at the catalytic centre. The major drawback of the three-metal ion mechanism is that the catalytically relevant positions I and III have never been observed occupied by divalent metal ions simultaneously [159].

In addition to the ambiguity in the number of divalent metal ions that are essential for catalysis, the identity of the general base that stabilizes the attacking nucleophile is also a matter of debate. Besides the 3′ phosphate that has been proposed in the one-metal ion mechanism to abstract a proton from the nucleophilic water molecule, several other candidates have been suggested. Lysine, present in the active site of most restriction enzymes, could deprotonate the water molecule if its pK_a is reduced by ~3 pH units. On the one hand, it is difficult to rationalize such a drop of the lysine pK_a in the proximity of the negatively charged sugar-phosphate backbone and the negative side chains of the carboxylates in the active site. On the other hand, a positively charged lysine can reduce the free energy of deprotonation by interacting favourably with the OH^- ion. Glu113 in BamHI in the corresponding position to lysine in the PD…D/ExK motif [154] has also been proposed to help in the formation of the nucleophile. Mutating the side chains that can participate in the nucleophile preparation step in EcoRV could not identify the general base unequivocally [117, 122]. Alternatively, besides protein residues, another water molecule can also be involved in the deprotonation step. The energetic analysis of all possible mechanisms of the nucleophile activation step has only been performed for BamHI using computer simulation approaches [161]. This study concluded that the deprotonation by a second water molecule connected to the bulk solvent is the most favourable mechanism, wherein the metal ion acts to lower the pK_a of a neighbouring water molecule. Recent molecular dynamics simulations on the active site of EcoRI support this hypothesis [118].

As mentioned above, Type II restriction endonucleases (with the few exceptions mentioned) have the same catalytic motif, the PD…D/ExK motif (in BamHI lysine is replaced by glutamic acid; in BglII by glutamine; both could function like lysine to position the attacking nucleophile by a hydrogen bond). The main problem is whether cleavage by restriction endonucleases can be described by a uniform catalytic mechanism in spite of the conflicting structural and biochemical data. In an attempt to develop a general catalytic model, some fundamental considerations will be introduced for consistent analysis of the different catalytic schemes. The catalytic effect of restriction enzymes will first be defined, and then the dependence of the catalytic effect on divalent metal cations will be investigated separately for the two reaction steps (generation of the nucleophile, stabilisation of the pentavalent transition state). In addition to rationalizing the divalent metal ion requirement of restriction endonucleases, this approach also helps to identify the main catalytic factors, including the number of essential divalent metal ions needed for the cleavage reaction. In the following we will use the assumption that phosphodiester hydrolysis by restriction endonucleases follows an associative pathway with the rate-limiting step being the attack of the nucleophile on the phosphate instead of the dissociation of the 3′-hydroxy group [162].

The schematic energy diagram for phosphodiester hydrolysis catalysed by a restriction enzyme compared to the reaction in water is shown in figure 5. The values of the reference reaction were deduced as follows: the free energy of the activation of the nucleophile is obtained as

$$\Delta G = 2.3RT\,[\mathrm{p}K_a(H_2O)\text{-}\mathrm{p}K_a(B)]$$

where pK_a(B) corresponds to the pK_a value of the general base that can be an amino acid residue or another water. In this case a glutamate has been considered. Since we want to examine only the difference between the enzyme and solution environment, the same reactants are considered in water as in the enzyme, and the free energy of bringing them into the same solvent (reacting) cage has been added to the free energy of the solution reaction [163]. The activation energy for proton transfer in water is taken from the work of Guthrie [160]. The activation energy for the nucleophilic attack on the phosphorous by a hydroxide ion is taken from the same reference [160].

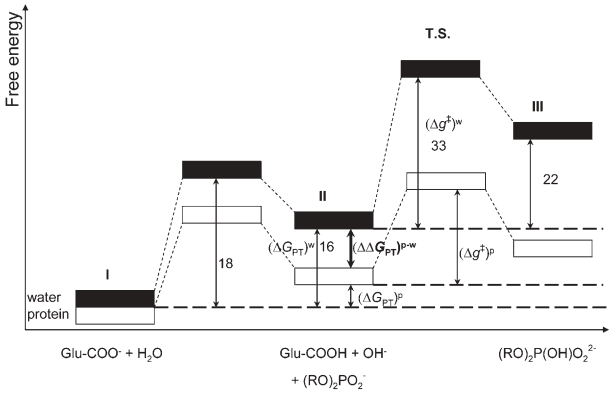

Figure 5. Free-energy profile of phosphodiester hydrolysis in water (black) and 'on' the protein (hatched). Assuming an associative mechanism, two reaction steps, the deprotonation of the water molecule to generate the nucleophile (state II) and the nucleophilic attack (leading to formation of a pentavalent intermediate, state III) are considered. The total activation energy ($\Delta G^{\ddagger}$) is obtained as the sum of the free energy of proton transfer (ΔG_{PT}) and the activation free energy of the nucleophilic attack ($\Delta g^{\ddagger}$). Assuming the same mechanism for the solution reaction, the catalytic effect is defined as the difference between these terms in protein and water, respectively: $(\Delta\Delta G^{\ddagger})^{p\text{-}w} = (\Delta\Delta G_{PT})^{p\text{-}w} + (\Delta\Delta g^{\ddagger})^{p\text{-}w}$. The numbers refer to a possible general base mechanism, the involvement of glutamic acid in the first step. The magnitudes (in Rcal/mol) are derived from [160].

Following the assumption that the attack of the nucleophile on the scissile phosphate is the rate-limiting step (associative mechanism), the total activation energy of the reaction is composed of two terms:

$$\Delta G^{\ddagger} = \Delta G_{PT} + \Delta g^{\ddagger}$$

where ΔG_{PT} is the free energy of proton transfer, and $\Delta g^{\ddagger}$ is the free energy of activation of the nucleophilic attack. In water, ΔG_{PT} is 21 kcal/mol if OH^- is involved to abstract the proton [164, 165] and the free energy of this step is reduced by 5 kcal/mol if a glutamate is employed as a base. The energy of activation of the attack of OH^- on the phosphate ($\Delta g^{\ddagger}$) has been measured as 33 kcal/mol [160]. The total free energy of activation ($\Delta G^{\ddagger}$) therefore is 54 kcal/mol for the stepwise reaction if OH^- is used as a reactant and 49 kcal/mol if a glutamate residue is used as the base. We have to note that in water the energy barrier of the hydrolysis of a phosphodiester by a neutral water molecule is more favourable, with a total activation barrier of $\Delta G^{\ddagger}$ = 36 kcal/mol [160]. To elucidate the enzymatic effect properly, the reference reaction must have the same mechanism as that of the enzyme. Hence the activation energy of the enzymatic reaction is calculated as in water as the sum of the free energy of the deprotonation and the activation energy of the nucleophilic attack steps:

$$(\Delta G^{\ddagger})^{p} = (\Delta G_{PT})^{p} + (\Delta g^{\ddagger})^{p}$$

It allows for a straightforward definition of the catalytic effect of the enzyme

$$(\Delta\Delta G^{\ddagger})^{p-w} = (\Delta G^{\ddagger})^{p} - (\Delta G^{\ddagger})^{w} = [(\Delta G_{PT})^{p} - (\Delta G_{PT})^{w}] + [(\Delta g^{\ddagger})^{p} - (\Delta g^{\ddagger})^{w}] = (\Delta\Delta G_{PT})^{p-w} + (\Delta\Delta g^{\ddagger})^{p-w}$$

where superscript *p* and *w* correspond to the protein-catalyzed reaction and the reaction in water, respectively. In addition to defining the catalytic effect quantitatively, this approach allows the separate analysis of the catalytic factors that facilitate the two reaction steps.

The rate of phosphodiester hydrolysis in restriction endonucleases is ~0.1 s^{-1} [16], giving an activation barrier of 19 kcal/mol. Hence the barrier for phosphodiester hydrolysis is reduced by ~30 kcal/mol compared to the corresponding reference reaction in water. The total catalytic effect of 30 kcal/mol is due to the stabilization of the attacking hydroxide ($\Delta\Delta G_{PT})^{p-w}$, as well as the stabilization of the pentavalent transition state ($\Delta\Delta g^{\ddagger})^{p-w}$). The relative magnitudes of these two effects regarding the reduction of the overall energy barrier of the reaction, however, are not available from direct measurements.

The most straightforward way to get an estimate of these relative contributions is to mutate side chains that interfere with either the generation of the nucleophile or the nucleophilic attack. Eliminating general base candidates in various enzymes decreased the catalytic rate by at least 3–4 orders of magnitude (e.g. EcoRV: [117, 166]), thereby increasing the overall energy barrier by 4–5.5 kcal/mol. These results either mean that these enzymes hardly optimize the energetics of the first reaction step or that the candidate amino acid residues do not act as general bases, and therefore the major contribution to nucleophile stabilization is provided by other factors, e.g. the proximal divalent metal ion.

Another way to assess the relative contributions of the two reaction steps to the reduction of the overall activation barrier is to determine the dependence of the $(\Delta\Delta G_{PT})^{p-w}$ and $(\Delta\Delta g^{\ddagger})^{p-w}$ terms on the divalent metal ion cofactors. Restriction endonucleases require divalent metal cations for catalysis, Mg^{2+} usually being the most efficient [167]. Ca^{2+}, however, inhibits the reaction, despite the fact it has been demonstrated to bind in a catalytically relevant position with the same octahedral geometry as Mg^{2+} (BamHI: [154]; EcoRV: [168, 169]), indicating that electronic rather than geometric factors are required for ion selectivity [170]. Most phosphodiester hydrolyzing enzymes are optimized for Mg^{2+}. Stapphylococcal nuclease is one of the few exceptions that use Ca^{2+} instead of Mg^{2+} to catalyze phosphodiester bond cleavage [171]. The metal ion selectivity of this enzyme has been rationalized by free-energy calculations with different metal ions [170], and the conclusions that emerged from this study will be applied for restriction endonucleases.

Let us start with the hypothesis that ΔG_{PT} and $\Delta g^{\ddagger}$ depend on the ion radius. It can be rationalized in the following way. The energetic contribution of the enzyme to the stabilisation of the 'intermediates' along the reaction pathway such as the OH^- nucleophile and the doubly charged pentavalent phosphorane are sensitive to the ion size. In general, smaller ions are more efficient in stabilizing both intermediates than larger metal ions. On the other hand, if the stabilization of OH^- is larger than that of the pentavalent phosphorane intermediate, it can lead to overstabilization or 'trapping' of the nucleophile by increasing the barrier for the subsequent nucleophilic attack. Therefore, the dependence of the overall energy barrier on the divalent metal ion is determined by the relative sensitivities of these two intermediates to the ion radius of the divalent metal ion (ionic radii for octahedral geometry are for Co^{2+}: $0.79^{(low\ spin)}$ – $0.89^{(high\ spin)}$ Å; Mn^{2+}: $0.81^{(low\ spin)}$ – $0.97^{(high\ spin)}$ Å; Mg^{2+}: 0.86 Å; Zn^{2+}: 0.88 Å; Ca^{2+}: 1.14 Å [172]). Among various ions tested (Ca^{2+}, Sr^{2+}, Ba^{2+}, Eu^{3+}, Tb^{3+}, Cd^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}) only Mn^{2+} and Co^{2+}, other than Mg^{2+}, supported DNA cleavage by PvuII [173]. In the following we will denote the energy of the nucleophile with E_I and that of the pentavalent state by E_{II}. Four possibilities can be envisaged that would favour divalent metal ions of different ionic radius: (i) E_I is more sensitive to the ion size than E_{II}; this would favour large ions due to nucleophile trapping problems; (ii) E_I is less

sensitive to the ion size than E_{II}; small ions would yield minimal $(\Delta G^{\ddagger})^{p}$; (iii) E_I is more sensitive to smaller ions and E_{II} is more sensitive to larger ions; ions of medium size would be preferred; and (iv) E_I is more sensitive to larger ions and E_{II} is more sensitive to smaller ions. The reaction would be fast either with small or with large metal ions (depending on the ratio of the ΔG_{PT} and $\Delta g^{\ddagger}$ terms) [170].

Thus, the metal ion selectivities are determined by the delicate balance between the catalytic effect of the enzyme on the generation of the nucleophile and the nucleophilic attack. Applying the above considerations to restrictions endonucleases allows concluding that Mg^{2+} is selected as the optimal ion, if the energy of the pentavalent phosphorane is more sensitive to the ion size than that of the OH^- nucleophile and most likely $(\Delta\Delta g^{\ddagger})^{p-w}$ is larger than $(\Delta\Delta G_{PT})^{p-w}$ (such that even for case (iv) a small ion would be favoured). The primary role of divalent metal cations in restriction endonucleases is therefore to stabilize the doubly charged pentavalent transition state and not to optimize the nucleophilic attack step. Why then is Ca^{2+} not as suited as Mg^{2+} to perform this task if the coordination geometries of the two metal ions are similar to each other? Efficient stabilization of the pentavalent transition state can be achieved if the charge transfer is small between the negative charges of the scissile phosphate and the divalent metal ion, i.e. the phosphate carries almost two negative charges and the metal ion carries two positive charges. In the presence of a strong charge transfer (in an extreme case a whole charge), the favourable electrostatic interactions that provide major stabilization of the transition state are substantially reduced compared to their values without the charge transfer. Depending on the enzymatic environment, it can lead to the decrease of the $(\Delta\Delta g^{\ddagger})^{p-w}$ term by more than 10 kcal/mol that results in an inactive enzyme. Computational studies in other enzymes demonstrated that Ca^{2+} ions are much more polarizable in this respect than Mg^{2+} ions [174]. Thus, if the enzymatic effect is more important for the nucleophilic attack than for the generation of the nucleophile (that is, if $(\Delta\Delta g^{\ddagger})^{p-w}$ is larger than $(\Delta\Delta G_{PT})^{p-w}$), charge transfer between Ca^{2+} and the doubly charged phosphorane would prevent catalysis.

The preference of restriction enzymes for Mg^{2+} ions indicates that the stabilization of the nucleophile is less dependent on the ion radius [case (ii), see above]. It suggests that OH^- may not be directly bound to the metal ion. Although direct ligation of OH^- would be energetically most favourable if only the optimization of ΔG_{PT} is considered, it increases the overall barrier by contributing unfavourably to the $\Delta g^{\ddagger}$ term by 'trapping' the nucleophile. Nevertheless, even if OH^- is not ligated to the cofactor, the metal ion is important to provide favourable contribution to the stabilization of the nucleophile. Note that tuning the pK_a of a general base (even by 4 pH units) can contribute to at most 5 kcal/mol to the $(\Delta\Delta G^{\ddagger})^{p-w}$ term, while the presence of a single metal ion can provide a favourable contribution of 8 kcal/mol [161]. A contribution by a second metal ion is considerably less, ~2 kcal/mol. It suggests that for the generation of the nucleophile the presence of one divalent metal ion is essential.

Restriction endonucleases need Mg^{2+} ions mainly for the nucleophilic attack. The contribution of the metal ion(s) to the reduction of the $(\Delta\Delta g^{\ddagger})^{p-w}$ term has not been quantified so far. The possible roles of the two metal ions have been probed in BamHI by substituting glutamic acid by lysine at position 77 and 113 [175]. The E113K mutant excludes metal ion A binding from the active site [154, 176] and results in the inactivation of the enzyme. The E77K mutation is likely to prevent metal ion B binding [154], but yields a functional enzyme with reduced catalytic rate when combined with a substitution nearby (R76K or P79T) [177]. These experiments suggest that metal ion A that is located in the proximity of the nucleophile and also coordinated to the scissile phosphate is a key factor in catalysis, while metal ion B, which ligates the O3′ leaving group is more variable, i.e. it can improve catalysis in some cases, but is not absolutely essential. The variability of the second metal ion site could also explain why the two catalytic metal ion sites have never been observed together in EcoRV [159].

Given the above considerations, the following general ideas are put forward to explain the catalytic efficiencies of restriction endonucleases. Based on the assumption that the reaction follows an associative pathway, the generation of the OH^- and the attack of the nucleophile on the scissile phosphate have to be considered in our energetic analysis. The dissociation of the leaving group has to be included in the analysis only if it becomes rate limiting, e.g. in mutant enzymes or in the absence of the metal cation. Otherwise, the ability of the metal ion to facilitate the dissociation step by lowering the pK_a of the water that protonates the 3′-oxyanion is not important for the total enzymatic effect. Thus, to provide a uniform catalytic scheme for the mechanism of DNA cleavage by restriction endonucleases, the energetic contribution of the metal ion(s) to the reduction of the free energy of the generation of the nucleophile $[(\Delta\Delta G^{\ddagger})^{p-w}]$ and to the stabilization of the pentavalent transition state $[(\Delta\Delta g^{\ddagger})^{p-w}]$ has to be determined. Only with such a quantitative analysis can a general catalytic model for restriction enzymes be developed.

The preference of restriction endonucleases for Mg^{2+} shows that during evolution these enzymes were optimized for stabilization of the pentavalent transition state rather than for generation of the OH^- nucleophile. Based on mutagenesis results it has been found that even in a case that is geometrically ideal for a two-metal mechanism (in BamHI), the individual contributions of the metal ions to the $(\Delta\Delta g^{\ddagger})^{p-w}$ term are not equal. Replace-

ment of the metal ion that is proximal to the nucleophile results in an inactive enzyme while upon replacement of the other the enzyme is still functional, indicating that only one of the metal ions is obligatory for catalysis. Computational analysis indicates that the free energy of proton transfer in the nucleophile generation step is also dominated by the contribution of one metal ion, even if two are present in the active site. These observations support the hypothesis that the presence of one metal ion is essential for restriction endonuclease action, whereas the identity of another group that increases the catalytic efficiency (for example a second divalent metal ion as in BamHI or, possibly, a histidine residue as in BsoBI) is more variable.

Based on the above results and considerations we propose that a uniform mechanism could apply for all restriction endonucleases of the PD…D/ExK family. This mechanism requires at least one divalent metal ion. The presence of a second divalent metal ion can improve catalysis, though the cleavage reaction can be performed in its absence as well. These considerations can rationalize why two metal ions are observed at the active site of several restriction endonucleases. The single and double metal ion mechanisms represent two alternative ways to facilitate phosphodiester bond hydrolysis [153]. If a single metal ion is present, it is responsible for catalyzing both reaction steps, stabilizing the OH^- nucleophile as well as the pentavalent transition state. To perform this task, the ion has to be able to move during the reaction. If two metal ions are available, the tasks can be divided, so less movement is required. In any case the attacking water molecule is proximal to the divalent metal ion held in place by two carboxylates of the PD…D/ExK motif and a main chain carbonyl (x of the PD..D/ExK motif). The proton from the attacking water is transferred to a water molecule nearby (which acts as general base) and eventually to the bulk solvent. The water molecules are hydrogen bonded to the 3′ phosphate and the lysine/glutamic acid/glutamine of the PD..D/ExK motif. The metal ion provides a favourable contribution to the stabilisation of the nucleophile. In contrast to mechanistic conclusions drawn in previous studies, divalent metal ion dependence suggests that the OH^- is not directly bound to the metal ion, as this would lead to trapping of the nucleophile and increase the barrier of the nucleophilic attack step. The key role of the divalent metal ion is to stabilize the pentavalent transition state. Leaving group stabilization is not rate limiting for the chemical step. After phosphodiester bond cleavage, the 3′ oxyanion is likely to associate itself with a divalent metal ion from the bulk solution (as seen in post-reactive complexes of BamHI [154], HincII [158] and NgoMIV [48]). Based on the preference of restriction endonucleases for Mg^{2+}, we hypothesize that these enzymes evolved to optimize the nucleophilic attack rather than the nucleophile generation step of the phosphodiester cleavage reaction. The above model is consistent with all experimental data known thus far.

If all type II restriction endonucleases of the PD…D/ExK family follow a similar mechanism in phosphodiester bond hydrolysis, why is it then that in co-crystal structures of some Type II restriction endonucleases with their substrate one divalent metal ion is seen while two divalent metal ions are in others? An alternative explanation to what is discussed above (variations of an essentially one-metal mechanism) could be that co-crystal structures with two divalent metal ions do not represent a pre-reactive complex, but rather an inhibited complex. A similar controversy existed with RNase H: crystallographic studies of the RNase H domain of HIV-1 reverse transcriptase revealed two Mn^{2+} ions in the active site [178], whereas the structurally related *Escherichia coli* RNase HI was shown to bind only one Mg^{2+} [179]. It was later shown for the *E. coli* enzyme that binding of one Mn^{2+} ion supports activity, while binding of a second Mn^{2+} ion inhibits activity [180, 181]. This is readily apparent from measurements of the Mg^{2+} or Mn^{2+} ion concentration dependence of the rate of phosphodiester bond cleavage, which show RNase HI activity to be optimal at Mg^{2+} or Mn^{2+} ion concentrations of 1–10 and 0.001–0.1 mM, respectively, and becomes progressively smaller at higher concentration. The Mg^{2+} dependence of the RNase activity of the *Methanococcus jannaschii* RNase HII displayed the same behaviour [182]. We have carried out similar experiments with a variety of different Type II restriction endonucleases, including EcoRI and NgoMIV, with essentially the same result [V. Pingoud, unpublished]. We conclude from these studies that the high concentrations of divalent cations used in co-crystallization or soaking experiments tend to lead to occupation of non-physiological Mg^{2+} ion binding sites, which would not be occupied in the pre-reactive complex at the physiological concentration (~1 mM for Mg^{2+}). Furthermore, the post-reactive state as seen in the crystal of BamHI [154], HincII [158] and NgoMIV [48] is not necessarily a good analogue of the transition state regarding metal ion occupancy: the additional negative charge at the newly formed 3′-phosphate is likely to attract a divalent ion, if present in high concentrations (e.g. 100 mM $MgCl_2$ used to determine the NgoMIV structure). The problem of charge neutralization is illustrated in the NgoMIV-product complex by an acetate ion bridging the two metal ions. Regarding the interpretation of electron densities during crystal structure analysis, it has to be considered that divalent metal ion binding may not be stoichiometric, meaning that two Ca^{2+} ions seen in two different positions in two individual protein molecules (each at a 1:1 stoichiometry) in the crystal can be mistakenly regarded as two divalent metal ions in two different positions bound to one protein molecule. Finally, the electron density attributed to a Ca^{2+} ion can be due to e.g. a Na^+ ion, as was recently reported

for a homing endonuclease of the LAGLIDADG family, I-CreI. In the original report two Ca^{2+} ions were seen in the pre-reactive complex of I-CreI, one per active site [183]. In a later paper three Ca^{2+} ions were identified and discussed in terms of a one-and-a-half-metal ion mechanism, i.e. a two-metal ion mechanism, in which one divalent metal ion is shared between two active sites [184, 185]. In the most recent publication, one of these Ca^{2+} ions was shown to be a Na^{+} ion [186]. It must be emphasized that in the post-reactive complex three Mn^{2+} ions were seen [184], which still is taken as evidence for a one-and-a-half-metal ion mechanism [186]. The metal-ion controversy concerning the LAGLIDADG homing endonucleases is similar to that for the PD…D/ExK restriction endonucleases, as also in this family of endonucleases co-crystal structures with one (e.g. PI-SceI [187]) or one and a half Ca^{2+} ions per active site (e.g. I-SceI [188]) were reported. We hope that this discussion makes it clear that additional biochemical and biophysical experiments are needed to settle the problem of how many divalent metal ions are directly involved in the catalysis of phosphodiester bond cleavage by restriction endonucleases of the PD…D/ExK family. As Horton and Perona correctly pointed out, 'A potential hazard is that restriction enzymes such as BamHI and BglI may remain understudied, and our understanding of them based on heavy reliance on X-ray data may include misinterpretations, because, given the appearance of a 'solved' mechanism, the rigorous kinetic data with which to make important correlations are never obtained' [159].

Evolution

As outlined above Type II restriction endonucleases are a very diverse group of enzymes. Most of them belong to the PD…D/ExK superfamily of endonucleases. With the exception of the catalytic motif, little sequence similarity has been observed between the more than 200 Type II restriction enzymes that have been sequenced to date. The few exceptions are isoschizomers that cleave the same sequence at the same position, e.g. EcoRI and RsrI (G/AATTC) [189], MthTI and NgoPII (GG/CC) [190], XmaI and CfrI (C/CCGGG) [191] and Cfr10I and Bse634I (R/CCGGY) [192]. Most isoschizomers, however, do not share significant sequence similarity. Limited sequence similarity has also been observed in some cases among restriction enzymes that recognize related sequences, e.g. EcoRI (G/AATTC) and MunI (C/AATTG) [193] and SsoII (/CCWGG) and PspGI (/CCNGG) [194]. Until about 1995 the generally accepted view was that restriction enzymes are not evolutionarily related. This began to change as crystal structures of Type II restriction endonucleases became available, demonstrating that these proteins to have a similar structural core that harbours the active site with the characteristic PD…D/ExK motif [76, 77, 195]. Furthermore, a statistical analysis revealed a significant correlation between the amino acid sequences ('genotype') of restriction enzymes and their recognition sequences and mode of cleavage ('phenotype'); these findings were interpreted as evidence for an evolutionary relationship among Type II restriction endonucleases [196]. It is clear now that Type II restriction enzymes of the PD…D/ExK superfamily evolved via divergent evolution [90], a process that was stimulated by the exchange of restriction-modification systems through horizontal gene transfer among bacteria and archaea [197].

It has been a great challenge to use the little sequence similarity present among Type II restriction endonucleases to unravel the evolutionary history of present day enzymes. That this can be done in principle using rapidly improving bioinformatic tools has been demonstrated for a group of restriction endonucleases that recognize related sequences and cleave DNA at the same position, namely SsoII (/CCNGG), PspGI (/CCWGG), EcoRII (/CCWGG), NgoMIV (G/CCGGC), Cfr10I (R/CCGGY) and their close relatives (SsoII: Kpn2kI/Ecl18kI/StyD4I/SenPI; Cfr10I: Bse634I/BsrFI) [198–200]. Intriguingly, it was recently shown that the evolutionary relationship between SsoII, PspGI, EcoRII, NgoMIV and Cfr10I can be extended to MboI which recognizes a very different sequence: /GATC [201]. Figure 6 illustrates the sequence alignment of these restriction endonucleases and presumptive relatives; this sequence alignment comprises the PD…D/ExK motif (or a variant of it: PD…S/TxK…E, present e.g. in Cfr10I, EcoRII, NgoMIV, PspGI and SsoII) and regions involved in DNA recognition. It is noteworthy that among these evolutionarily related restriction endonucleases there are Type IIP (SsoII, PspGI, MboI), Type IIE (EcoRII) and Type IIF enzymes (NgoMIV, Cfr10I), demonstrating that structural elements which can serve as effector domains (Type IIE) or tetramerization subdomains (Type IIF) can be acquired at various stages during evolution. With the structural information available on PD…D/ExK enzymes it is possible to construct a cladistic tree of these structures and to suggest hypothetical intermediates in the evolution of the PD…D/ExK enzymes [90].

In this context it is worth mentioning that the PD…D/ExK motif is not only characteristic of the majority of Type II restriction enzymes, but is also found among Type I, III and IV restriction enzymes [202] as well as other nucleases (table 1). It is certainly surprising that the PD…D/ExK motif is so dominating among restriction enzymes of all types, in particular as the core structure in which it is embedded is associated with different subdomains, domains and polypeptide chains that determine the type and subtype. One wonders why, for example, another catalytic motif, the $\beta\beta\alpha$Me-finger that is found in non-specific nucleases (such as the Serratia nuclease [203] and the apoptotic nucleases CAD and EndoG [204, 205]),

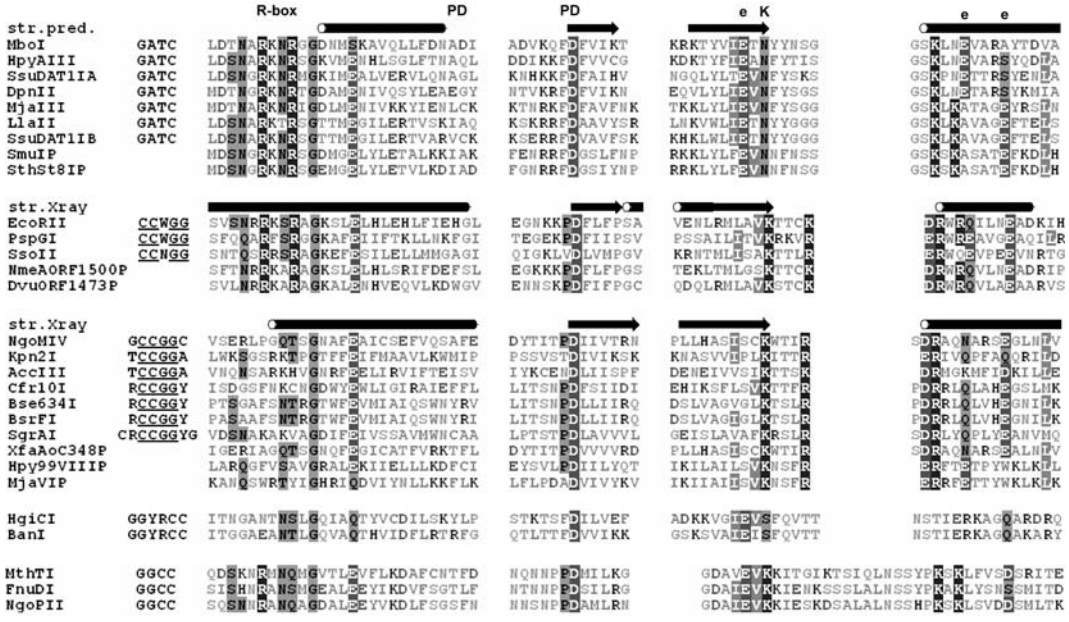

Figure 6: Alignment of 29 genuine and putative Type II restriction enzymes that share sequence similarity over a stretch of approximately 70 amino acid residues interrupted by two gaps of variable length. This region is part of the conserved core of the PD…D/ExK superfamily of Type II restriction endonucleases. In NgoMIV, for which a co-crystal structure is available, this region encompasses helices 3 and 7 and β-strands 2 and 3 that are involved in DNA recognition and cleavage. The involvement of this region in DNA binding and cleavage by Cfr10I and EcoRII is also apparent from crystal structures. For SsoII, PspGI and MboI, mutational analysis and protein-DNA cross-linking determined this region to be likewise important. The alignment is subdivided into five groups, based on the similarity of the amino acid sequence and the recognition sequence. Genuine Type II restriction enzymes are indicated by their recognition sequence; putative restriction enzymes have a postfix P in the name. Amino acid residues are shown in grey scale according to the similarity of their physico-chemical properties; conserved residues are highlighted. Residues that are important for DNA binding (R-box) and cleavage (PD…e.K…e…e) are shown above the alignment (adapted from [200]).

structure specific nucleases (such as T4Endo7 [206]) and homing endonucleases (such as I-PpoI [207, 208]) is hardly at all represented among restriction endonucleases. It is tempting to speculate that RM systems originated very early in evolution and that early restriction enzymes used the PD…D/ExK motif, which served its purpose and was therefore kept as the dominant catalytic motif among these enzymes. That this motif functions as a stabilization factor for the whole protein structure [209, 210] may be another reason for its conservation.

Synopsis

Restriction enzymes are of paramount importance for recombinant DNA work and efforts are under way to make them even more useful by expanding or changing their specificity. In addition, they have been model systems to study various aspects of protein-nucleic acid interactions: target site location, recognition, catalysis. There has been a lot of progress in this area, yet questions remain, in particular regarding the mechanism of catalysis. More recently, and stimulated by the fast progress made in deciphering the genomes of bacteria and archaea, restriction enzymes and their companion enzymes, the DNA methyltransferases, have attracted attention as model systems to understand the evolution of a large family of related enzymes. We anticipate exciting discoveries in this area.

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