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S-Adenosyl-L-methionine–Dependent Restriction Enzymes

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Restriction–modification (R–M) enzymes are classified into type I, II, III, and IV, based on their recognition sequence, subunit composition, cleavage position, and cofactor requirements. While the role of S-Adenosyl-L-methionine (AdoMet) as the methyl group donor in the methylation reaction is undisputed, its requirement in DNA cleavage reaction has been subject to intense study. AdoMet is a prerequisite for the DNA cleavage by most type I enzymes known so far, with the exception of R.EcoR124I. A number of new type II restriction enzymes belonging to the type IIB and IIG family were found to show AdoMet dependence for their cleavage reaction. The type III enzymes have been found to require AdoMet for their restriction function. AdoMet functions as an allosteric effector of the DNA cleavage reaction and has been shown to bring about conformational changes in the protein upon binding.

Keywords restriction–modification enzymes, methyltransferase, endonuclease, AdoMet, DNA cleavage

The ATP-activated form of methionine, S-adenosyl-L-methionine (AdoMet), is the major and most commonly used methyl group donor in all biological systems. Its role in methylation has been well studied in a number of systems, including DNA methylation, small molecule methylation, protein methylation, and RNA methylation.

I. CHEMISTRY OF AdoMet

Figure 1 shows the structure of AdoMet. AdoMet methyl group is bound to a charged sulfur atom, which thermodynamically destabilizes the molecule and makes the relatively inert methylthiol of the methionine moiety very reactive (Walsh, 1979). Two arms, each highly functional, flank the methyl center with its sulfonium leaving group. During the methyl transfer reaction no chemical changes occur in the two functional arms, yet enzyme binding to these arms can serve several complementary purposes. Firstly, AdoMet can be bound in the active site with its methyl group so oriented as to permit highly efficient methyl transfer according to the expected mechanism of a colinear transfer with inversion of stereochemistry at methyl group. Second, the attachment of the two arms of the structure to the enzyme permits effective spatial reorganization by the enzyme, if this is required along the reaction path. Further, the initial interactions of the enzyme with the two arms are further enhanced in the catalytic transition state(s). The net increase in favorable interactions constitute net transition state stabilization and therefore contribute to catalysis (Takasugawa *et al*., 1998).

II. AdoMet AS COFACTOR

AdoMet has several chemical advantages over other methyl group donors. AdoMet appears to be three orders of magnitude more reactive towards polarizable nucleophiles (N, O, and S) than methylated folates (Cantoni, 1975). The $\Delta G^{o\prime}$ for AdoMet hydrolysis in methylation is -17 kcal/mol; in comparison, the $\Delta G^{o\prime}$ for ATP hydrolysis to ADP+Pi is -7.3 kcal/mol (Boyer *et al*., 1975; Matsuno-Yagi & Hatefi, 1988).

AdoMet has myriad roles in metabolism, including methyl group donor in many reactions, propylamine donor in the biosynthesis of polyamines spermine and spermidine, and a noncovalent role as corepressor of the methionine biosynthesis regulon in *E. coli* and *S. typhimurium* (Cantoni, 1952; Greene *et al*., 1973; Hobson & Smith, 1973; Saint-Girons *et al*., 1988; Tabor & Tabor, 1984). Recently it has been implicated in controlling gene expression by binding riboswitches, structural domains embedded within the noncoding sequences of certain mRNAs that serve as metabolite-responsive genetic control elements (Winkler *et al*., 2003). Mutants deficient in AdoMet synthetase map to *metK*, which encodes AdoMet synthetase. Temperature-sensitive null mutants of *metK* have

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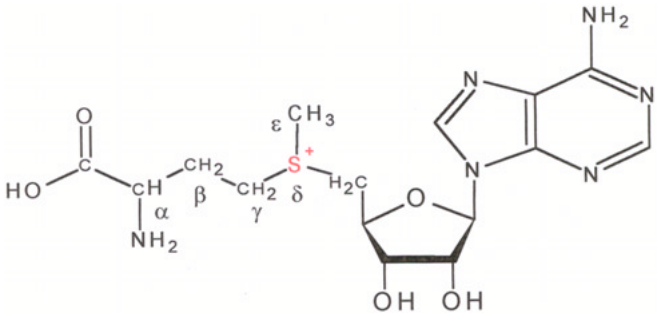

FIG. 1. S-Adenosyl-L-methionine.

dramatic reduction in both intracellular AdoMet levels and AdoMet synthetase and display novel growth characteristics (Satishchandran *et al.*, 1990).

One of the most well studied functions of AdoMet is as a methyl group donor. DNA methylation is brought about by a group of enzymes, DNA methyltransferases that are a part of restriction–modification (R–M) systems in prokaryotes. Methylation of DNA results in protection of the genome from restriction enzymes, which are a defense against infecting phages. Methylation of DNA predominantly occurs at cytosine and adenine residues. Cytosine methylation can take place at the C5 position or at the N4 amino group, whereas adenine methylation occurs at the N6 amino group (Wilson & Murray, 1991). All three forms of methylation are found in prokaryotes. DNA methyltransferases (MTases) can be broadly be classified into two groups, the exocyclic amino MTases comprising of the N4-cytosine and N6-adenine MTase families and the endocyclic carbon MTases, comprising the C5-cytosine MTase family (Dryden, 1999). All DNA MTases share a set of conserved motifs, of which motif I has been identified to be a part of AdoMet binding site and motif IV was found to be involved in methyl group transfer. The amino-MTases have been further subdivided into three groups, namely α, β, and γ, which are characterized by distinct linear orders for conserved motifs (Malone *et al.*, 1995).

R–M systems thus comprise of two opposing functions—methylation that protects the genome and restriction that results in cleavage of the same. Methylation is catalyzed by the modification (Mod) subunit and DNA cleavage by the restriction (Res) subunit of these enzymes. The large number of R–M systems presently known has necessitated the need to pool them together based on similarities and differences.

III. CLASSIFICATION OF R–M SYSTEMS

Originally, the RM systems were classified into three types, I, II, and III, based on the number and organization of subunits, regulation of their expression, cofactor requirements, enzymatic mechanism, and sequence specificity (Wilson & Murray, 1991). With the addition of further subtypes, a new nomenclature (Roberts *et al.*, 2003) has been proposed to include all the "novel" systems, intermediate to the old ones and with new structural features. Based on the requirement of AdoMet for their cleavage function, the R–M enzymes can be classified into AdoMet-dependent and AdoMet-independent R–M systems. The salient features of these enzymes are listed in Table 1. A number of excellent reviews have described various aspects of R–M systems (Dryden *et al.*, 2001; Halford, 2001; Pingoud & Jeltsch, 2001; Redaschi & Bickle, 1996a; Szczelkun, 2000, 2002). This review will focus on the AdoMet-dependent R–M enzymes.

TABLE 1
Types of restriction-modification enzymes

Feature	Type I	Type II	Type III	Type IV
Structural				
Subunits	Three different	Two identical	Two different	Two different
Enzyme activity	Endonuclease, methyltransferase ATPase	Endonuclease or methyltransferase	Endonuclease, methyltransferase ATPase	Endonuclease GTPase
Biochemical				
Cofactors for DNA cleavage	ATP, AdoMet, Mg^{2+}	Mg^{2+}	ATP, Mg^{2+} (AdoMet)	Mg^{2+}, GTP
Methylation	AdoMet, Mg^{2+}	AdoMet	AdoMet, Mg^{2+}	—
Recognition sequence	Asymmetric, bipartite	Usually symmetric	Asymmetric	Bipartite, methylated
Cleavage site	Random, atleast 1000 bp from recognition site	At or near recognition site	25–27 bp from recognition site	Between methylated bases at multiple positions
DNA translocation	Yes	No	Yes	Yes

IV. AdoMet-DEPENDENT DNA R–M SYSTEMS

DNA cleavage by the type I and type III restriction enzymes requires AdoMet or is stimulated by it (Table 1). Some families of type II restriction enzymes are also known to require AdoMet for their function or are stimulated by it.

A. Type I R–M Enzymes

By far the most complex among the R–M systems, type I enzymes have three different subunits that form a multifunctional enzyme and catalyze both restriction and modification of DNA. The first to be discovered, they are found mainly in *E. coli* and *Salmonella* species and are apparently widespread in the eu- and archea-bacteria (Roberts & Macelis, 2001). Functional enzymes have been discovered in *Bacillus subtilis* (Xu *et al*., 1995), *Citrobacter freundii* (Daniel *et al*., 1988), *Klebsiella pneumoniae* (Lee *et al*., 1997; Valinluck *et al*., 1995), various strains of *Lactococcus lactis* (Schouler *et al*., 1998), Mycoplasma *pulmonis* (Dybvig & Yu, 1994), *Neisseria gonorrhoeae* (Piekarowicz *et al*., 2001), *Pasteurella haemolytica* (Highlander & Garza, 1996), *Staphylococcus aureus* (Sjostrom *et al*., 1978) and *Streptococcus thermophilus* (Solow & Somkuti, 2001).

These enzymes recognize asymmetric sequences split into two domains of specific sequence, one 3–4 bp long and another 4–5 bp long, separated by a nonspecific spacer of defined length, 6–8 bp. Cleavage occurs a considerable distance from the recognition sites, rarely less than 400 bp away and up to 7000 bp away. Adenosyl residues are methylated, one on each strand of the recognition sequence.

i. Families of Type I Systems. Among all the bacteria, three genes involved in type I restriction and modification are *hsd*S, *hsd*M, and *hsd*R (where *hsd* denotes host specificity for DNA). The *hsdS* gene is necessary for both restriction and modification and is responsible for recognition of the DNA sequence specific for the system. The *hsdM* gene is necessary for modification, and the *hsdR* gene, together with the other two genes, is required for restriction (Boyer & Roulland-Dussoix, 1969; Glover & Colson, 1969; Hubacek & Glover, 1970). Structural genes for many type I systems from *E. coli*, *Salmonella*, and *Citrobacter freundii* have been cloned and sequenced with results that corroborate the results of genetic analysis (Sain & Murray, 1980; Gough & Murray, 1983; Loenen *et al*., 1987; Cowan *et al*., 1989; Kannan *et al*., 1989; Price *et al*., 1987).

In enteric bacteria these systems have been examined genetically and biochemically and grouped into four families, IA, IB, IC, and ID, based on genetic complementation, DNA hybridization, and antibody cross-reactivity (Barcus *et al*., 1995). The salient features and representative members of these families are listed in Table 2.

ii. Hsd Subunits. The type I restriction enzymes are complex oligomeric proteins comprised of HsdS

TABLE 2
Type I restriction-modification systems

Families	Members	Enzyme/recognition sequence*		Salient features
A	*E. coli, Klebsiella* and *Salmonella* species	EcoKI	AAC(N)$_6$GTGC	Chromosomally encoded, allelic
		EcoAI	TGA(N)$_8$TGCT	
		StySBI	GAG(N)$_6$RTAYG	
		EcoID	TTA(N)$_7$GTCY	
B	*E. coli, C. freundii*	EcoAI	GAG(N)$_7$GTCA	Chromosomally encoded, allelic
		CfrAI	GCA(N)$_6$GTGG	
		StySKI	CGAT(N)$_7$GTTA	
C	*E. coli, Klebsiella* and *Neisseria* species	EcoR124I	AA(N)$_6$RTCG	*E. coli* conjugative plasmids, nonallelic
		EcoR124/3I	GAA(N)$_7$RTCG	
		EcoDXXI	TCA(N)$_7$RTTC	
		KpnBI	CAAA(N)$_6$RTCA	
		NgoAV	GCA(N)$_8$TGC	
D	*E. coli, Klebsiella* and *Salmonella* species	StySBLI	CGA(N)$_6$TACC	
		KpnAI	GAA(N)$_6$TGCC	
		EcoR9I		
	Lactococcus lactis	LldI		Novel specificity

*N, any nucleotide; R, either purine; Y, either pyrimidine.

(∼50 kDa), HsdM (∼50–60 kDa) and HsdR (∼140 kDa) subunits. The modification enzyme contains products of *hsdM* and *hsdS* genes in a stoichiometry reported to be 1S:1M for type IA enzyme, EcoBI; 1S:2M for the type IA enzyme, EcoKI (Dryden *et al*., 1993); and EcoR124I (Lautenberger & Linn, 1972; Taylor *et al.*, 1992) for the type IC enzyme. The restriction enzyme contains products of all the three genes, *hsd*R, *hsd*M, and *hsd*S. Each enzyme is a large oligomeric complex of relative molecular weight, ∼400,000 to 500,000, which in the presence of Mg^{2+}, ATP, and AdoMet functions as an endonuclease. The restriction enzyme also catalyzes the transfer of methyl groups from AdoMet to DNA that has either unmethylated or hemimethylated target sequences (Vovis *et al*., 1974). The organization of these subunits to form the R–M enzyme is illustrated in Figure 2.

HsdS. The HsdS subunit recognizes specific target sequences on the DNA and is responsible for the specificity of type I R–M systems. There are two large nonhomologous regions (of about 150 amino acids) at the N-terminal and C-terminal halves of the subunit. The two variable regions form two distinct folded domains, each recognizing one-half of the bipartite recognition sequence (Kannan *et al*., 1989). The N-terminal domain of 150 aminoacids has been shown to specify the trinucleotide component of the target sequence and the C-terminal domain recognizes the tetra-pentanucleotide component (Cowan *et al*., 1989). The amino and carboxy TRDs (target recognition domains) of the HsdS subunit are in inverted orientation within the R–M complex, forming a structure with twofold rotational symmetry (Kneale, 1994; Burckhardt *et al*., 1981). The conserved sequence at the N terminus forms part of the region that contacts HsdM. Each TRD fits into the major groove to recognize that the DNA and the modification subunits are arranged on either side of the specificity subunit, allowing them to encircle the DNA and gain access to the methylation targets (Kneale, 1994; Dryden *et al*., 1995). The conserved regions of the HsdS

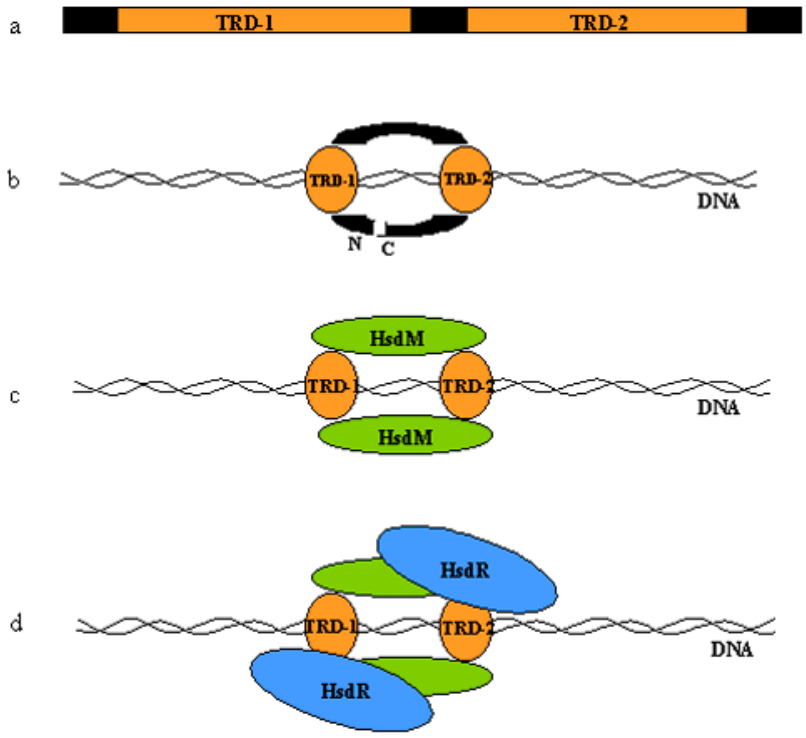

FIG. 2. Model of the assembly of type I R–M enzyme subunits. (a) The HsdS subunit comprises three short conserved domains (in black) and two large variable domains (in white) called target recognition domains (TRDs). (b) TRD-1, the amino terminal TRD, recognizes and binds to the first half of the target site (tri/tetra nucleotide sequence), whereas TRD-2, the carboxy terminal TRD, recognizes and binds to the second half of the recognition site (tetra/penta nucleotide sequence). (c) The methylase is formed by the addition of two HsdM subunits, one on either side of the HsdS subunit, away from the helical axis of the DNA. (d) A stable endonuclease complex is formed on unmethylated DNA in the presence of ATP by the addition of two HsdR subunits to the methylase (M2S1) + AdoMet complex. The presence of the DNA translocation catalytic site on the HsdR subunit indicates that the endonuclease contains secondary DNA binding sites, one on each HsdR subunit (Adapted from Bourniquel & Bickle, 2002).

polypeptides have structural roles associated with them, maintenance of relative positions of the two TRDs, and specific association with other components of the R–M complex (Murray, 2000).

HsdM. Like HsdS, HsdM is essential for restriction as well as modification. The HsdM subunits are responsible for binding the methylation cofactor AdoMet, determining the methylation status of the target sequence, and carrying out methylation of adenine bases, probably achieved through a base flipping mechanism where the target base is rotated 180° out of the DNA helix into the enzyme catalytic site (Mernagh *et al*., 1998; Roberts & Cheng, 1998). The target adenines are located in the 5′ part of target sequence on the top strand and 3′ part of the target sequence within the bottom strand, respectively. The preferred substrate is hemi-methylated DNA, though unmethylated DNA can also get methylated, albeit slowly and only in the presence of all the three subunits. Methylation complex made up of two HsdM subunits along with a HsdS subunit is required for methylation activity (Janscak & Bickle, 1998; Dryden *et al*., 1993; Taylor *et al*., 1992). HsdM subunit has the characteristic motifs common to all adenine MTases (Loenen *et al*., 1987; Klimasauskas *et al*., 1989). These include motif I (D/E/SXFXGXG), which is responsible for AdoMet binding, and motif IV (N/DPPF/Y/W), which is involved in catalysis (Willcock *et al*., 1994). These are found in the central third of the subunit and form a domain. Proteolysis of the Mod subunit showed that the amino-terminal domain contains a region that influences the enzyme specificity for hemimethylated DNA (Kelleher *et al*., 1991). The carboxy-terminal is responsible for binding HsdM to HsdS, and its removal prevents its interaction with the rest of the protein (Cooper & Dryden, 1994; Powell *et al*., 2003). Levels of sequence identity of HsdM within the family are >90%, while between the families are much lower, except at the conserved motifs (Sharp *et al*., 1992). The heterodimer M_1S_1 can only bind DNA, whereas the addition of the second Mod subunit to form M_2S_1 confers MTase activity. The dimeric form has weaker affinity for *Eco*KI target sequence than the trimer and slightly less ability to discriminate against other DNA sequences (Powell *et al*., 1998). The functional form of MTase, M_2S (Suri & Bickle, 1985; Patel *et al*., 1992; Taylor *et al*., 1992; Dryden *et al*., 1993) catalyzes the transfer of methyl group from AdoMet to the N-6 position of a specific adenine residue in each part of the recognition site, i.e., one on each strand of DNA (Nagaraja *et al*., 1985a). DNA binding is enhanced in the presence of AdoMet, and it probably plays a role in distinguishing between unmodified and modified DNA. Footprinting experiments indicate that the two HsdM subunits are located on either side of DNA helical axis rather than lying along the helical axis (Powell & Murray, 1995).

HsdR. HsdR subunit is the third protein of the type I R–M complex and is responsible for endonuclease activity. The addition of two HsdR subunits to the MTase (M_2S_1) completes the type I enzyme ($R_2M_2S_1$), such as in the case of EcoKI (Dryden *et al*., 1997), StySPI and StySBI (Nagaraja *et al*., 1985b), EcoR124I (Janscak & Bickle, 1998), and EcoR124II (Dreier & Bickle, 1996).

The HsdR subunit is poorly conserved. Near the N-terminus is a proteolytically defined region of 400 amino acids containing an amino acid motif very similar to that found in the catalytic site of type II restriction endonucleases (Titheradge *et al*., 1996). The C-terminal region is required for binding to the MTase core (Davies *et al*., 1999). It is more likely that the major contacts are between the R subunit and S subunit, as it has been found that interactions between R and M subunits are weak (Dryden *et al*., 1997). The Walker A and B motifs, as well as five others found in the family of DEAD box DNA and RNA helicases (Gorbalenya & Koonin, 1991; Murray *et al*., 1993; Titheradge *et al*., 1996; Webb *et al*., 1996) implicated in ATP binding, hydrolysis, and DNA translocation are found in the intervening region. However, there is no evidence yet that a helicase mechanism involving strand separation is actually operational in type I enzymes, although DNA movement (translocation) is clearly revealed (Berge *et al*., 2000; Davies *et al*., 1999; Dreier *et al*., 1996; Dryden *et al*., 1997; Ellis *et al*., 1999; Firman & Szczelkun, 2000; Janscak *et al*., 1999; Rosamond *et al*., 1979; Szczelkun *et al*., 1996).

iii. Mechanism of Action. The ability of the type I enzymes to catalyze the opposing reactions of restriction and modification is intriguing. Enzyme activity can best be understood by subdividing it into distinct stages (Hadi *et al*., 1975; Yuan *et al*., 1975; Yuan & Hamilton, 1984)—enzyme activation, DNA binding and recognition, enzyme action, and loss of restriction function (nuclease activity) (Figure 3).

The early steps of the reaction mechanism are common to both methylation and restriction. Cleavage occurs at an undefined point 1000–7000 bp away from the recognition sequence. Two or more unmethylated target sites are required for cleavage of linear DNA, though a single site containing substrates can be cleaved at higher (10-fold) enzyme concentrations. (Murray *et al*., 1973; Studier & Bandyopadhyay, 1988; Janscak *et al*., 1996; Dreier *et al*., 1996). Single-strand scission precedes cleavage of duplex DNA (Meselson & Yuan, 1968). These enzymes do not turn over as nucleases (Eskin & Linn, 1972; Yuan *et al*., 1975), and hence two molecules are required to complete double-strand cleavage. Modified DNA is not a substrate for the reaction (Linn & Arber, 1968). Topology of DNA substrate plays a role in restriction as proposed by

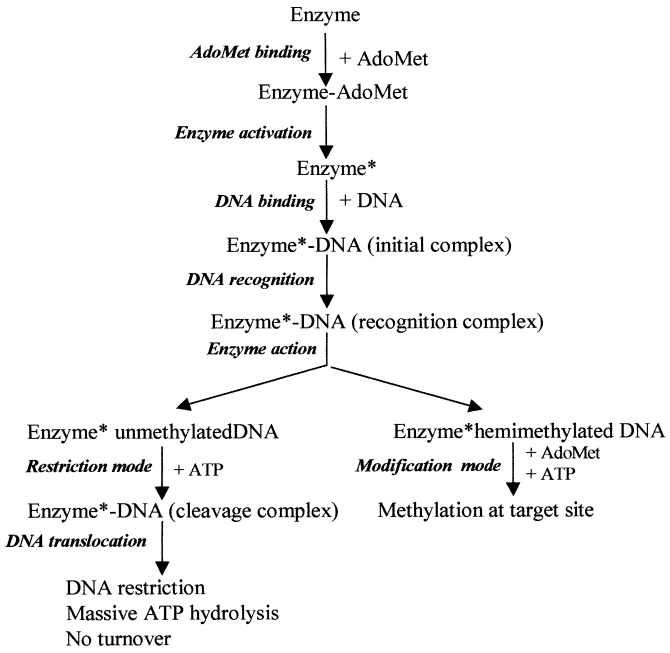

FIG. 3. Reaction mechanism of type I R–M enzymes.

Janscak *et al*. (1996). Cleavage pathways for linear and supercoiled substrates appear to be different (Szczelkun *et al*., 1996).

AdoMet binding is the first, rapid step of the reaction pathway. Having bound AdoMet, transition to an activated enzyme form is slow. The enzyme is proposed to have two types of AdoMet binding sites: effector and methyl donor sites. Both sinefungin (an analog of AdoMet) and AdoMet bind to the former, while the affinity of AdoMet binding is greater at the methyl donor site. The effector sites are accessible to AdoMet (or sinefungin) before the enzyme is bound to DNA but not afterwards. The methyl donor sites are able to interact with free AdoMet only after the formation of recognition complexes. *HsdS* mutation allows normal AdoMet binding but efficiently blocks enzyme activation (Brack *et al*., 1976).

DNA recognition involves formation of a nonspecific complex between DNA and the activated enzyme species and subsequent formation of a specific complex at the recognition site, the recognition complex. Methylation status of DNA and interaction with ATP commits the complex to its final course of action. A fully methylated site results in the enzyme falling off, while hemi-methylated DNA stimulates enzyme into the modification mode. Unmethylated DNA shifts the enzyme to a 'restriction mode,' resulting in DNA cleavage. After the cleavage event, the enzyme is structurally altered and loses its ability to cleave DNA. It remains bound to the DNA (Yuan *et al*., 1975; Rosamond *et al*., 1979) and continues to hydrolyze ATP (Eskin & Linn, 1972; Yuan *et al*., 1972; Dreier & Bickle, 1996). The alteration in the enzyme possibly prevents it from turning over.

ATP has dual roles—as cofactor in the DNA cleavage reaction and as substrate in the ATP hydrolysis reaction. ATP is required in two distinct steps in EcoKI-mediated DNA cleavage (Bickle *et al*., 1978). It induces a conformational change in the enzyme that aids in discriminating between modified and unmodified recognition sites. ATP is required to translocate the DNA past the enzyme till the cleavage site is reached, and cleavage occurs only in the presence of ATP hydrolysis (Dryden *et al*., 2001). Translocation generates highly twisted loop structures (Yuan *et al*., 1980), eventually resulting in DNA cleavage. ATP hydrolysis continues even after restriction and is probably due to alteration of the enzyme, yielding a species that remains stably bound to the DNA (Yuan *et al*., 1975; Eskin & Linn, 1972; Horiuchi *et al*., 1974; Rosamond *et al*., 1979; Dreier & Bickle, 1996). Recognition of certain undefined cleavage regions results first in the cleavage of one strand followed rapidly by a second cut in the complementary strand at a site opposite the first (Meselson & Yuan, 1968).

Several models have been proposed to explain the mechanism of DNA cleavage by type I R–M systems. According to the collision model proposed by Studier and Bandhyopadhyay (1988), the type I endonuclease binds to its specific recognition site and translocates DNA towards itself simultaneously from both directions. This generates expanding loops of DNA coming out from the enzyme–DNA complex. DNA cleavage occurs at the site where convergently translocating enzyme molecules meet. The study carried out with linear substrates was extended to circular substrates and to linear substrates with a single site. In the latter case, cooperative interactions between bound and excess free enzymes would result in cleavage (Szczelkun *et al*., 1996).

The expanding loops of DNA have been visualized by electron microscopy (Rosamond *et al*., 1979; Yuan *et al*., 1980) and were not observed in the absence of ATP. Observations, using atomic force microscopy, of EcoKI bound to DNA containing two target sequences in the absence of ATP failed to show individual protein molecules bound to the two sites (Ellis *et al*., 1999; Berge *et al*., 2000). Instead, the proteins dimerized and collapsed the DNA into a small volume. Addition of ATP caused translocation of DNA until cleavage occurred, when DNA could not be looped any further. This "tracking" model, formally equivalent to the collision model, overcomes the problem of requiring the two translocating complexes to move closer together while dragging expanding loops of DNA behind themselves through a cytoplasm crowded with other macromolecules (Goodsell, 1991).

Another model explaining mechanism of action of type I enzymes suggested that the enzyme tracks along the major groove of DNA, and consequently no collision would

occur between two enzyme molecules. DNA cleavage was purported to be triggered by excessive buildup of positive supercoils inducing either a halt or stall in the translocation process (Szczelkun *et al*., 1996). Thus according to this model, positively supercoiled DNA would be cleaved faster than relaxed DNA. Janscak and Bickle (2000) disproved this by showing that positive supercoiled DNA was in fact cleaved much more slowly than relaxed DNA. Excessive negative supercoiling also resulted in decreased rates of cleavage. While supercoiling is not responsible for triggering DNA cleavage, there is convincing evidence for the role of physical barriers, such as those represented by Holliday junctions, inducing DNA cleavage (Janscak *et al*., 1999). Noncovalently bound Lac repressor is merely displaced by the tracking endonuclease and has no effect on DNA cleavage (Dreier *et al*., 1996). Evidence supports DNA cleavage occurring whenever the translocating endonuclease is stalled (Dreier *et al*., 1996). A kinetic model of translocation has been proposed to support the stalling-induced DNA cleavage model (Szczelkun, 2002).

Based on all of the above observations, yet another model has been proposed for DNA translocation and cleavage by type I endonuclease (Bourniquel & Bickle, 2002). The endonuclease binds to its recognition site on unmethylated DNA and dimerizes either by an intramolecular process between adjacent sites or intermolecularly with an enzyme bound to another DNA substrate molecule. In the presence of ATP, each HsdR subunit of the two complexes translocates DNA in a bidirectional process (Firman & Szczelkun, 2000; Studier & Bandyopadhyay, 1988). Thousands of base pairs are thus translocated and DNA cleavage occurs. The HsdR subunits are free to rotate around or detach from the methylase core (M_2S_1), which remains bound to the target sequence (Davies *et al*., 1999; Janscak & Bickle, 2000). Continued ATP hydrolysis occurs even after DNA cleavage (Dreier & Bickle, 1996; Yuan *et al*., 1972; Eskin & Linn 1972; Horiuchi *et al*., 1974), and the number of ATP molecules hydrolyzed is far in excess of the number of double-strand breaks (Yuan *et al*., 1972). Further, the continued presence of DNA was required to sustain ATP hydrolysis even after cleavage was complete (Dreier & Bickle, 1996; Eskin & Linn, 1972). It was proposed that during cleavage, the enzyme is converted from a form capable of nuclease activity to one catalyzing massive hydrolysis of ATP (Yuan *et al*., 1972). An altruistic view suggested by Bickle (1993), states that when a phage-infected cell hydrolyzes so much ATP it will not survive, ensuring that the population remains pure (uninfected by phages) at the cost of a few individuals.

iv. Role of AdoMet in the Cleavage Reaction. The endonuclease catalyzes DNA cleavage and ATP hydrolysis. AdoMet, Mg^{2+}, and ATP are essential cofactors of the endonuclease. The role of cofactor AdoMet in complex formation is purely steric; it has been shown to alter the contact points on DNA that were necessary for restriction (Powell & Murray, 1995). ATP induces a conformational change in the enzyme that aids in the discrimination between modified and unmodified recognition sequences (Bickle *et al*., 1978). AdoMet cannot be replaced by any of a number of related compounds (Meselson & Yuan, 1968). S-adenosylethionine or 5′-methylthioadenosine inhibit the restriction reaction (Eskin & Linn, 1972; Figure 4).

Type I enzymes EcoKI and EcoBI have an absolute requirement for AdoMet during restriction (Meselson & Yuan, 1968; Hadi *et al*., 1975). AdoMet was not consumed in these reactions but was found to be a positive allosteric effector. The role of AdoMet in *Eco*KI-mediated DNA restriction has been studied using the wild-type enzyme and two mutant enzymes, one defective in restriction and the other lacking the ability to recognize the cognate DNA sequence. These enzymes were found to hydrolyse ATP in an AdoMet-dependent and DNA-independent manner (Hadi & Yuan, 1974).

The enzyme interacts with AdoMet in the absence of DNA, suggesting that the binding of AdoMet to the enzyme is the first step in the reaction. Activation is a slow process, suggesting that the mere binding of cofactor to the enzyme was not sufficient; rather it triggers a slow transition of the enzyme to an activated state (Bickle *et al*., 1978). AdoMet binding also results in a much slower release of the enzyme from DNA (Janscak *et al*., 1996). The enzyme subsequently cleaves the double-stranded DNA without any further requirement for free AdoMet. These results have led to the proposal of a sequence of events leading to DNA restriction (Figure 3).

It is known that all type I restriction enzymes characterized so far require AdoMet for DNA cleavage (Bourniquel & Bickle, 2002), with the exception of R.EcoR124I, which was found to require only ATP and Mg^{2+} for its DNA cleavage activity. However, AdoMet stimulated the ATPase and DNA cleavage activities of R.EcoR124I. AdoMet was found to influence DNA binding, probably by bringing about a conformational change, which is not an absolute requirement for DNA binding (Janscak *et al*., 1996). The methylation state of the recognition site of M.EcoKI does affect the conformation of the protein at the DNA interface, and AdoMet may play an important role in discrimination between unmodified and modified DNA (Powell & Murray, 1995). Extrapolating this observation to the restriction enzyme, it seems likely that AdoMet signals the switch between restriction and modification modes via a conformational change with altered base contacts (Powell & Murray, 1995). AdoMet acts as an allosteric activator for Type I restriction enzymes (Hadi *et al*., 1975). Such a function for the methyl donor could serve to regulate restriction and prevent suicide under conditions of methionine starvation, where unmethylated recognition sequences may

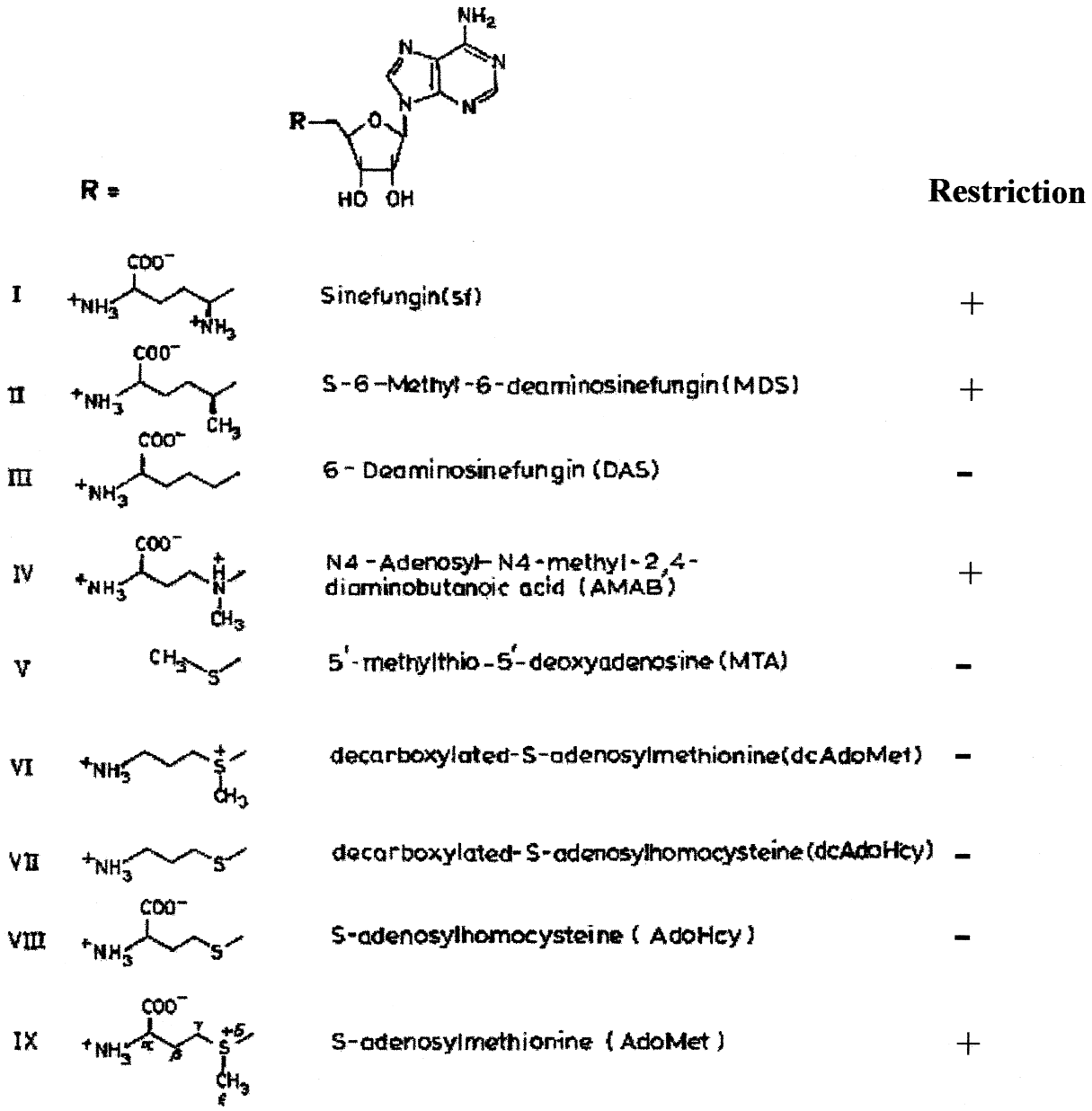

FIG. 4. Analogs of AdoMet.

arise on the cellular chromosome (Lark & Arber, 1970; Meselson *et al*., 1972; Modrich, 1979).

B. Type II R–M Enzymes

These are the simplest among the R–M systems and have two functions of restriction and modification, carried out by different enzymes. The orthodox type II enzymes recognize palindromic sequences 4–8 bp long and cleave within the sequence in a reaction requiring Mg^{2+}. Methylation occurs on both the strands in the sequence with a preference for hemi-methylated DNA, using AdoMet as the methyl group donor. The cognate type II methylases belong to any one of the families of N6, N4, or C5 MTases.

The type II family is further classified into several subtypes based on their gene organization, cleavage pattern, and structural organization. The type IIG and type IIB subfamilies include members that require AdoMet for their activities or are stimulated by it (Table 3).

i. Type IIG. Eco57I and GsuI are restriction enzymes, where the Res and Mod subunits are fused to form a single polypeptide that has both MTase and endonuclease

TABLE 3
AdoMet dependent Type II restriction enzymes

Enzyme	Recognition sequence
Type IIG	
BseMII	5′CTCAG(N)10/8
BsgI	5′GTGCAG(N)16/N14
Eco57I	5′CTGAAG(N)16/N14
GsuI	5′CTGGAG(N)14/16
MmeI	5′TCCRAC(N)20/18
Type IIB	
BaeI	10/15 AC $(N)_4$GTAYC 12/7
BcgI	10/12 CGA$(N)_6$TGC 12/10
BplI	8/13 GAG$(N)_5$CTC 13/8
Bsp24I	8/13 GAC$(N)_6$TGGN 12/7
HaeIV	7/13 GAY$(N)_5$RTC 14/9

*N, any nucleotide; R, either purine; Y, either pyrimidine.

(REase) functions associated with it. AdoMet stimulates the endonuclease function, and cleavage occurs away from the recognition sequence (Petrusyte *et al*., 1988). A number of enzymes belong to this group, such as Bce83I, BseMII, BseRI, BsgI, BspLU11III, Eco57I, GsuI, MmeI, and Tth111II (Table 3). Some of these have been well studied and are discussed below.

Eco57I. Eco57I consists of two separate proteins; a multifunctional enzyme with REase and MTase activities and the other with only MTase activity. While the MTase requires only AdoMet, the MTase activity associated with the multifunctional enzyme requires AdoMet and Mg^{2+} for methylation activity. Cleavage of substrate occurs 16/14 bp 3′ of the recognition sequence, 5′-CTGAAG (Petrusyte *et al*., 1988). Methylation by R.Eco57I occurs only on the top strand of the recognition sequence, while the methyltransferase transfers methyl groups onto the recognition sequence on both the top and bottom strands (Janulaitis *et al*., 1992). Certain properties of the multifunctional enzyme, such as stimulation of restriction in the presence of AdoMet, incomplete digestion of DNA, and methylation of only one strand make them seem like the type III enzymes. However, unlike the type III enzymes, these enzymes do not require ATP for restriction, and methylation is not stimulated by it.

Other type IIG members. GsuI cleaves DNA 16/14 nucleotides away from the recognition sequence, 5′-CTGGAG. DNA cleavage is stimulated by AdoMet and is complete both in the absence and presence of AdoMet. This suggests an absence of methylation activity (Janulaitis *et al*., 1983; Petrusyte *et al*., 1988). Unlike Eco57I, a second enzyme with MTase activity has not been detected in the case of GsuI.

BseMII recognizes the sequence 5′-CTCAG and cleaves DNA 10/8 bases away from it. AdoMet and Mg^{2+} are required for cleavage and DNA is completely cleaved. ATP does not affect the endonucleolytic activity of the enzyme. Analogs of AdoMet such as S-adenosyl homocysteine (AdoHcy) and sinefungin (Figure 4) could replace AdoMet as cleavage cofactor, indicating that binding of cofactor is essential for cleavage (Jurenaite-Urbanaviciene *et al*., 2001). MmeI cleaves DNA 20/18 bases away from the sequence, 5′-TCCRAC (where R strands for purine). The enzyme comprises a single polypeptide, which catalyzes restriction and methylation activities, and a second component, a methyltransferase, which methylates both adenines in the recognition sequence on top and bottom strands. Hemimethylated DNA, where the adenine of the top strand is methylated, does not inhibit DNA cleavage by R.MmeI (Tucholski *et al*., 1998). DNA cleavage is incomplete and is enhanced in the presence of AdoMet and sinefungin.

ii. Type IIB. These enzymes are a subset of type II enzymes that cleave DNA on either side of their recognition sequence and require Mg^{2+} and AdoMet for cleavage. Cleavage results in the release of a fragment about 30 bp long, including the recognition site. BcgI (Kong *et al*., 1993), Bsp24I (Degtyarev *et al*., 1993), CjeI and CjePI (Vitor & Morgan, 1995) and BaeI (Sears *et al*., 1996) are some of the members of this family.

BcgI. The genes encoding BcgI have been cloned and sequenced. The *bcg*IA gene codes for a 71.6 kDa protein, which is homologous to adenine-specific MTases. The *bcg*IB gene codes for a 39.2 kDa protein. Neither protein can cleave or methylate DNA by itself, but together they form a complex of A_2B capable of both of these functions (Kong *et al*., 1994). DNA cleavage requires Mg^{2+} and AdoMet. Interestingly, though AdoHcy (Figure 4) cannot substitute for AdoMet, sinefungin can. The enzyme recognizes the sequence CGA$(N)_6$TGC and cleaves DNA releasing a 34 bp fragment that can be methylated (Kong *et al*., 1993). Methylation requires only AdoMet, although Mg^{2+} stimulates it. ATP does not enhance methylation. Site-directed mutagenesis was carried out to investigate the relationship between AdoMet binding and BcgI DNA cleavage/methylation activities. Most substitutions in the AdoMet binding pocket abolished both methylation and cleavage activities, indicating that AdoMet binding is an early common step required for both cleavage and methylation. Mutagenesis in the catalytic motif, D/NPPY (motif IV), resulted only in the methylation step being affected and not the AdoMet binding step or cleavage reaction thereafter. This clearly indicates that the methylation and cleavage reactions diverge after AdoMet binding (Kong & Smith, 1997).

Other type IIB enzymes. BplI, unlike the other BcgI-like enzymes, recognizes a symmetric interrupted sequence and cleaves double-stranded DNA upstream and downstream of its recognition sequence. A bifunctional enzyme, it shows low cleavage activity in the presence of Mg^{2+} alone, which is greatly stimulated in the presence of AdoMet, sinefungin, or AdoHcy. The interesting feature of this enzyme is that in the presence of AdoMet activity increases >100-fold but specific cleavage is incomplete. Surprisingly, in the presence of AdoHcy cleavage is complete. ATP is not required for cleavage (Vitkute *et al*., 1997). BaeI is composed of two proteins and has properties similar to those of BcgI. Cleavage requires both Mg^{2+} and AdoMet, and AdoHcy cannot replace AdoMet (Sears *et al*., 1996).

The structural difference and differential requirement of cofactors for the activities of these enzymes has been extended to suggest their possible evolution. Fusion of the Mod and Res subunits of type III enzymes, accompanied by deletion of the helicase domains, would probably have resulted in the type IIG enzymes (Dryden, 1999). Type IIG could have given rise to other forms, such as type IIB. Activation of DNA cleavage by AdoMet is suggestive of Res-Mod fusion, resulting in the formation of these new families of enzymes (Raghavendra & Rao, 2003). It has been suggested that the progenitor of R.Eco57I was generated by the fusion of Mod and Res subunits of a type III endonuclease (Janulaitis *et al*., 1992).

C. Type III R–M Enzymes

Type III R–M enzymes are hetero-oligomeric, multifunctional proteins composed of two subunits, products of *res* and *mod* genes. The Mod subunit is considered as a functional analog of the complex formed by the modification (M) and specificity (S) subunits of type I systems.

There are four well-known type III R–M systems. These are EcoP1I encoded by the *E. coli* prophage P1 (Arber & Dussoix, 1962), EcoPI5I encoded by the plasmid p15B in *E. coli* strain $15T^{-}$ (Arber & Wauters-Willems, 1970), HinfIII from *Haemophilus influenzae* Rf (Piekarowicz *et al*., 1974), and StyLTI found in most *Salmonella* species (Bullas *et al*., 1980). An isoschizomer of HinfIII, named HineI was isolated from *Haemophilus influenzae* Re (Piekarowicz, 1982). Besides these, putative R–M systems have been found by sequence alignment and homology search in diverse organisms such as *Moraxella catarrhalis* (Seib *et al*., 2002) and *Helicobacter pylori* (Kong *et al*., 2000; Lin *et al*., 2001), among others. The only type III R–M system that has been characterized in Gram-positive bacteria is the LlaFI system from *Lactococcus lactis*, which has two open reading frames organized as an operon. Restriction by LlaFI requires ATP and Mg^{2+} and is stimulated by AdoMet (Su *et al*., 1999). A genomic library fragment of *Bacillus cereus* has been found to share 30% homology with a part of the EcoP1I R–M system (Hegna *et al*., 1992). This R–M system, designated BceS1, is the first chromosomally encoded type III R-M system from gram-positive bacteria (Hegna *et al*., 2001) and requires Mg^{2+} and ATP for its endonuclease activity. AdoMet has no effect on the cleavage reaction.

Type III enzymes differ markedly from type I enzymes with respect to their recognition sequences. They recognize short 5–6 bp long asymmetric DNA sequences and cleave 25–27 bp downstream to leave short, single-stranded 5′ protrusions (Bickle, 1993; Rao *et al*., 2000; Dryden *et al*., 2001). They require the presence of two inversely oriented (→◄) unmethylated recognition sites for restriction to occur (Meisel *et al*., 1992). Methylation proceeds irrespective of the number and orientation of the sites. The peculiar feature of these enzymes is that they methylate only one strand of the DNA, at the N-6 position of adenosyl residues. Thus, newly replicated DNA always has one strand methylated, and this is sufficient to protect against restriction (Rao *et al*., 2000). The type III enzymes and their recognition sequences are shown in Table 4.

i. Res-Mod Operon. The only type III systems well-characterized genetically are EcoP1I and EcoPI5I. These two systems complement each other (Arber & Wauters-Willems, 1970). Type III enzymes are coded by two contiguous structural genes called *mod* and *res* (Iida *et al*., 1983). The *mod* gene product recognizes the DNA sequence specific for the system and is a modification methyltransferase. The *res* gene product alone has no enzymatic activity but, in association with the *mod* gene product, is restriction proficient. All the genetic information required to specify the R–M system is contained in a 5kb contiguous DNA segment carrying two genes (Iida *et al*., 1983), the 2.2 kb *mod* gene and 2.8 kb *res* gene. Several observations implied that the *mod-res* operon was transcribed as a single unit (Arber *et al*., 1975; Hümbelin *et al*., 1988; Sharrocks & Hornby, 1991). The nucleotide sequences of StyLT1 *mod* and *res* have been determined.

TABLE 4
Type III restriction-modification systems

Type III system	Bacterial strain	Recognition sequence
EcoP1I	*E. coli* phage PI	5′-AGACC-3′
EcoP15I	*E. coli* plasmid 15B	5′-CAGCAG-3′
Hinf III	*Haemophilus influenzae* Rf	5′-CGAAT-3′
HineI	*Haemophilus influenzae*	5′-CGAAT-3′
StyLTI	*Salmonella typhimurium* LT7	5′-CAGAG-3′
Hpy790545P	*Helicobacter pylori*	5′-CTGCAG-3′
Phmod/Phres	*Pasteurella haemolytica* A1	5′-CACAG 3′

The two adjacent open reading frames were found 12 bp apart and revealed homologies with known type III enzymes, EcoP1I, EcoPI5I and *Bacillus cereus* chromosome (Dartois *et al*., 1993). Sequencing of LlaFI revealed two adjacent open reading frames organized as an operon, with significant similarity to type III restriction and modification subunits. LlaFI Mod is more closely related to HinfIII Mod, while the Res protein has homology to EcoP1I and StyLTI Res proteins, the only Res subunits whose sequence is known (Su *et al*., 1999).

The type III R–M enzymes belong to the β subfamily of N6 adenine methyltransferases. They have the nine characteristic motifs of methyltransferases, which include motif I, the AdoMet binding pocket (FXGXG), and motif IV, the catalytic region (S/D/N (PP) Y/F) (Malone *et al*., 1995) (Figure 5). The Mod subunit is responsible for DNA recognition and methylation. The carboxy-terminal portion of the Mod subunit has the AdoMet binding region (Ahmad & Rao, 1994).

DNA cleavage and ATP hydrolysis are functions of the Res subunit. The Res subunit lacks DNA recognition function and is active only in conjunction with the Mod subunit. The Res subunit contains the so-called DEAD-box motif present in the helicase superfamily II of DNA and RNA helicases (Gorbalenya & Koonin, 1991). The members of the DEAD family have seven conserved motifs (motifs I, IA, and II–VI), including a DEAH motif (motif II) (Figure 5). Based on the amino acid sequence comparisons between the restriction subunits of EcoKI and EcoP1I and those of RNA and DNA helicases, Gorbalenya and Koonin (1991) suggested that type III enzymes could have helicase activity. These enzymes have an endonuclease domain at the carboxy-terminal region of the Res subunit termed "PD . . . (D/E)XK," for a very weakly conserved signature of the active site of several nucleases, including type II restriction endonucleases. It is implicated in metal binding, a prerequisite for DNA cleavage by most restriction enzymes (Pingoud & Jeltsch, 2001) (Figure 5). The charged residues form the catalytic triad and are implicated in metal ion-mediated catalysis (Viadiu & Aggarwal, 1998).

ii. Mechanism of Action. Survival of T7 phage (in which all the EcoP15I sites are in the same orientation), in contrast to T3 phage (wherein some of the EcoP15I sites are present in opposite orientation) on *E. coli* harboring the EcoPI5I system demonstrated the requirement of two sites in opposite orientation for restriction. A similar scenario exists in case of M13, which has 3 sites for EcoPI5I in the same orientation and was found refractory to cleavage by the EcoPI5I restriction enzyme. However, when any one of these sites was altered so that at least two adjacent sites were in a head-to-head orientation, DNA was linearized (Meisel *et al*., 1992). Cleavage by these enzymes requires two sites in opposite orientation and occurs 25–27 bp downstream from one of the recognition sequences, in an ATP- and Mg^{2+}-dependent reaction (Meselson & Yuan, 1968; Haberman, 1974; Reiser & Yuan, 1977; Bachi *et al*., 1979; Hadi *et al*., 1979). As in case of type I enzymes, ATP hydrolysis and DNA translocation are necessary for DNA restriction by the type III enzymes.

Earlier it was shown that restriction activity depends on the structure of the substrate DNA (Piekarowicz &

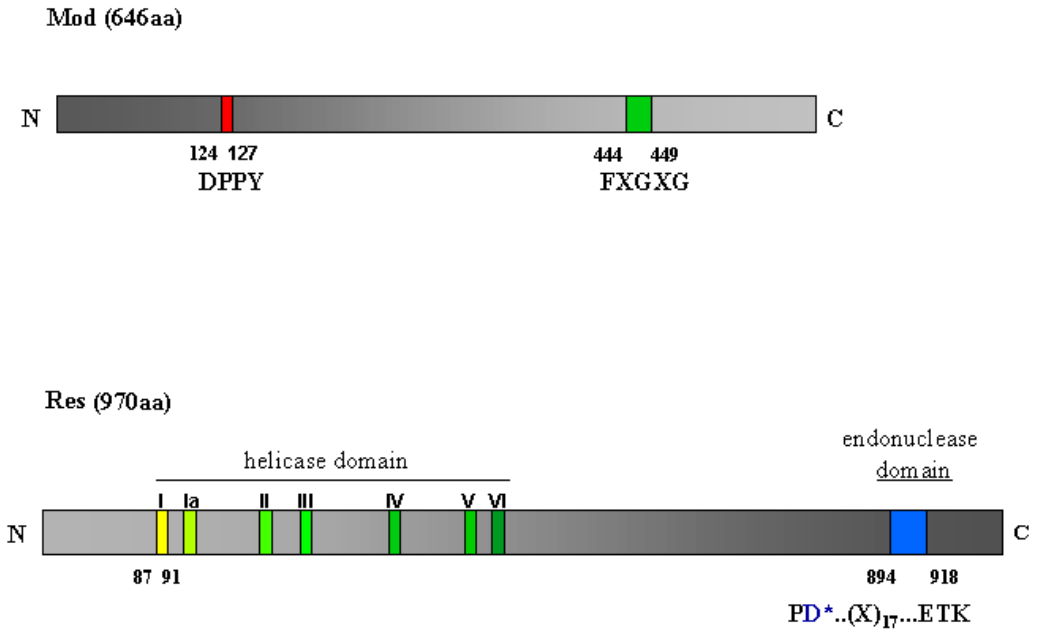

FIG. 5. Domain organization of Mod and Res subunits of EcoP1I R–M enzyme.

Brzezinski, 1980). Linear DNA was cleaved at a slower rate than supercoiled DNA. Piekarowicz (1984) found that at least three recognition sites are required for DNA cleavage to occur as enzyme molecules bound at several target sites result in more efficient cleavage. Methylation of the substrate, however, is independent of site orientation.

Meisel *et al.* (1995) proposed a collision-cleavage model for the type III enzymes, based on their results with R.EcoPI5I. Enzyme molecules bound at two adjacent inversely oriented sites translocate DNA past them in a reaction driven by ATP hydrolysis. DNA translocation is required for the two enzyme-substrate complexes to come in close apposition, which results in the formation of cleavage-competent complexes (Meisel *et al.*, 1995; Mücke *et al*., 2001). When two enzyme molecules converge in the correct head-to-head orientation, conformational changes activate the complex, resulting in DNA cleavage. This step requires ATP hydrolysis. The top strand is cleaved by one of the Res subunits from one enzyme molecule, while the bottom strand is cleaved by one of the Res subunits derived from the second endonuclease in the collision complex. Since these enzyme complexes collide in head-to-head configuration, the resulting complex would presumably have rotational symmetry (Janscak *et al*., 2001). A nonspecific molecular barrier such as a bound Lac-repressor, which would halt DNA translocation, is not sufficient to evoke DNA cleavage (Meisel *et al*., 1995). Type III enzymes do not rely on stalling to activate cleavage (Janscak *et al*., 2001).

The presence of helicase motifs in the Res subunit and the importance of these motifs for ATP-dependent DNA-cleavage reaction suggest that the mechanism of DNA translocation of type III enzymes may be related to DNA-unwinding brought about by the action of helicase. However, localized unwinding of DNA was not revealed during restriction (Saha & Rao, 1997). This could be due to small or transient changes in supercoiling that are relieved faster than in the case of type I enzymes. Methylation of recognition site could facilitate dissociation (Dryden *et al*., 2001). Recent studies have shown that an intact recognition site on the cleaved DNA sequesters the restriction enzyme and decreases the effective concentration of the enzyme. Presence of an exonuclease releases the sequester enzyme molecules, rendering them available for multiple rounds of catalysis to occur (Raghavendra & Rao, 2003).

One of the fundamental differences between the type I and type III enzymes is the mode of cleavage. In case of type I enzymes, cleavage does not occur at a defined distance from the sites but at a point where the translocating enzyme complexes collide. Type III enzymes cleave DNA at a defined distance from the recognition sequence. Which one of the two oppositely oriented sites is selected for cleavage is random. Unlike type I enzymes, type III restriction enzymes cut DNA exclusively on one side of their recognition site (Bächi *et al*., 1979; Hadi *et al*., 1979), suggesting an asymmetric architecture of the DNA–enzyme complex. Another contrasting feature of the two families of enzymes is that type I enzymes can either restrict or modify the DNA but cannot do both simultaneously, as can type III enzymes (Yuan & Hamilton, 1984).

Recently it has been shown that DNA translocation is not required for initiating DNA cleavage in case of substrates with adjacent head-to-head– or tail-to-tail–oriented target sites. As the two sites are further apart, cleavage rates increased for the sites, which are oriented head to head and decreased for the tail-to-tail–oriented sites. Single-site substrate cleavage was seen in case of R.EcoPI5I that was not a trans effect (Mücke *et al*., 2001). At high enzyme concentrations, DNA linearized by cleavage at one of the two potential sites could further be cleaved to generate two linear products. This "secondary cleavage" was observed only in the presence of potassium ions. Plasmids with two directly repeated sites and with a single site were cleaved under similar conditions (Peakman *et al*., 2003).

DNA substrate possessing one EcoP1I and one EcoP15I site in head-to-head configuration was specifically cleaved in the presence of these two enzymes (Kunz *et al*., 1998). Thus, two different enzymes can functionally cooperate in DNA cleavage (Janscak *et al*., 2001). This explains why cleavage of DNA by these enzymes is never complete and how freshly replicated DNA in cells harboring these systems escape restriction. As only one strand of the target site is methylated, after a round of replication fully unmethylated DNA will be produced. All of the sites, which are fully modified, are protected from cleavage because they will essentially be in the same orientation.

iii. Role of AdoMet in Cleavage Reaction. *In vivo* experiments of Lark and Arber (1970) with methionine analogue ethionine suggested that P15$^-$ lysogenic cells do not require AdoMet for host-specific DNA breakdown. *In vitro*, AdoMet was not absolutely required for DNA cleavage activity of purified EcoP15I, but it stimulated the rate of reaction. Thus, while the absence of AdoMet resulted in extensive cleavage, the presence of AdoMet resulted in incomplete cleavage, presumably due to methylation (Reiser & Yuan, 1977).

Certain aspects of the reaction mechanism of the type III R–M enzymes, particularly those of R.EcoP15I, have been investigated (Reiser & Yuan, 1977; Yuan & Reiser, 1978; Yuan *et al*., 1980). The cleavage reaction of HinfIII was studied by Brzezinski & Piekarowicz (1982). Binding of enzyme to its DNA substrate can occur in the absence of the cofactors, AdoMet or ATP (Yuan & Reiser, 1978; Brzezinski & Piekarowicz, 1982). The rate of complex formation is rapid and unaffected by the absence of AdoMet. Both AdoMet and ATP were found to be allosteric effectors in the restriction reaction. AdoMet was found to affect

the interaction of ATP with the complex. Enzyme-DNA complexes formed in the presence of AdoMet were cleaved at lower ATP concentrations and showed faster kinetics of cleavage on the addition of ATP than enzyme-DNA complexes formed in the absence of AdoMet (Yuan & Reiser, 1978; Brzezinski & Piekarowicz, 1982). The role of AdoMet in the reaction catalyzed by the type III enzymes is different from type I enzymes, where it has been shown to activate the enzyme in order for it to bind DNA specifically (Hadi *et al*., 1975). Earlier studies indicated that AdoMet is not required for cleavage, but merely stimulates the reaction catalyzed by the type III enzymes (Haberman, 1974; Reiser & Yuan, 1977). The role of AdoMet in the reaction catalyzed by these enzymes is more complex, since restriction and modification are competing reactions.

Meselson and Yuan (1968) first purified restriction activity from an *E. coli* K (P1) lysogen. The requirement for Mg^{2+}, ATP, and AdoMet was established and they first suggested that ATP and AdoMet were allosteric effectors. This was further established for EcoP1I (Habermann, 1974) and EcoPI51 (Yuan & Reiser, 1978). Studies with HinfIII system showed that restriction reaction does not strictly require AdoMet but is stimulated by it (Kauc & Piekarowicz, 1978; Brzeziski & Piekarowicz, 1982). HinfIII enzyme has been found to copurify with AdoMet bound to it, which is lost slowly upon storage (Piekarowicz & Brzesinksi, 1980). HinfIII-mediated cleavage of DNA was found to be incomplete, suggesting the possibility of bound AdoMet that methylates the recognition site, rendering it unsuitable for cleavage. It is possible that inside the cell most of the HinfIII molecules are in the AdoMet-bound form (Hinf III*), and the release of AdoMet takes place during purification or upon storage of the enzyme (Piekarowicz & Brzeziski, 1980). AdoMet acts as an allosteric effector and the cleavage reaction is stimulated by the addition of external AdoMet, even though the HinfIII* form has some AdoMet bound to it (Brzeziski & Piekarowicz, 1982). It can bind further AdoMet *in vitro*, which is referred to as $HinfIII^{+}$. Cofactors ATP and Mg^{2+} are a prerequisite for cleavage by HinfIII, BceS1 (Hegna *et al*., 2001), and LlaFI (Su *et al*., 1999). AdoMet has no effect on cleavage by BceS1 while LlaFI is stimulated by it. Effect of AdoMet is reminiscent of allosteric activation; progress curves of the reaction were sigmoidal for both EcoP1I and EcoPI5I (Yuan & Reiser, 1978; Bist *et al*., 2001).

Earlier work on the survival of genetically closely related bacteriophages T3 and T7 indicated that AdoMet is an obligatory or facultative cofactor of some host-controlled restriction enzymes (Krueger *et al*., 1975). In T3 phage, the 0.3 gene encodes an AdoMet hydrolase (Ado MTase) that hydrolyses AdoMet. Analysis of efficiency of plating (e.o.p) of the phages T3, T7, and $T3sam^{-}$ on P1-lysogenic cells clearly indicated that T3 was partially protected from EcoP1I restriction by its Ado MTase due to degradation of intracellular AdoMet. More recently, it has been demonstrated that EcoP15I and EcoP1I restriction enzymes cleave DNA specifically only in the presence of AdoMet (Bist *et al*., 2001). Using analogs of AdoMet (Figure 4), it was shown that other than AdoMet only those analogs with groups at the carboxyl group and ε-position, sinefungin, S-6-Methyl-6-deaminosinefungin and N4-Adenosyl-N4-methyl-2, and 4-diaminobutanoic acid could support DNA cleavage by R.EcoP1I and R.EcoP15I (Bist *et al*., 2001). While AdoMet was bound to the purified R.EcoP15I, EcoP1I restriction enzyme purified without bound AdoMet and therefore requires exogenous addition of AdoMet in the reaction for DNA cleavage. More importantly, the apoenzyme form of R.EcoP15I, which was completely inactive, showed similar DNA cleavage activity as the holoenzyme upon addition of AdoMet (Bist *et al*., 2001). HinfIII have been reported to exist as AdoMet-bound forms. The HinfIII* form (AdoMet bound Hinf III) was found to gradually convert to an AdoMet-free form upon storage (Piekarowicz & Brzezinski, 1980).

Specific conformational changes upon binding AdoMet were seen in the case of EcoP1I and EcoP15I. These conformational changes include differences in mobilities of the holo- and apoenzyme on nondenaturing polyacrylamide gels. Significantly, preincubation of the apoenzyme with AdoMet resulted in an enzyme form that had the same mobility as the holoenzyme, clearly suggesting that upon AdoMet binding to the enzyme a more compact structure is attained that migrates faster than the apoenzyme form. UV, fluorescence, and CD spectra of the apoenzyme and holoenzyme were distinctly different upon addition of cofactor to the apoenzyme form; the spectra resembled that of the holoenzyme, indicating conformational changes upon AdoMet binding (Bist *et al*., 2001).

The effector role occurs through the Mod subunit by enhancement of DNA binding and/or subunit–subunit interactions. Recently, it has been suggested that the DNA cleavage reaction is sensitive to the buffer conditions, i.e., favored in the presence of potassium (K^{+})-based buffers and disfavored in the presence of sodium (Na^{2+})-based buffers. The requirement for AdoMet in the cleavage reaction has been shown to be alleviated in the presence of potassium, and cleavage proceeds efficiently in the absence of cofactor. It has therefore been suggested that potassium and AdoMet elicit a similar allosteric response (Peakman *et al*., 2003).

AdoMet has been proposed to act as a "specificity factor," preventing DNA cleavage by enzymes bound to the inappropriate arrangement of sites. Halford (2001) reported that R–M systems communicate between multiple recognition sequences to enhance the specificity of molecular recognition. Promiscuous cleavage is seen in the presence

of potassium and not in the presence of sodium. Secondary cleavage seen in the presence of potassium was prevented upon addition of AdoMet. Although AdoMet is not strictly required for DNA cleavage, its presence maintains an unconditional requirement of two sites in inverse orientation, thus eliminating promiscuous cleavage. Substrates containing a single recognition site could also be cleaved, at elevated concentrations of enzyme (Peakman *et al*., 2003).

In the presence of 2M NaCl, the Res and Mod subunits of EcoP15I enzyme can be separated. With the availability of the mutant Mod subunit, which does not show significant AdoMet binding (Ahmad & Rao, 1996), restriction enzyme was reconstituted with the wild-type Res subunit and the mutant Mod subunit. Such a restriction enzyme had the same oligomeric status as the apoenzyme and was not able to cleave DNA. These results clearly argue for the fact that a functional Mod subunit (which binds AdoMet) is required to associate with the Res subunit to form a functional enzyme (Bist *et al*., 2001). A similar observation was reported with the BcgI R–M enzyme wherein most substitutions of conserved residues forming the AdoMet binding pocket abolished methylation activity and cleavage activity (Kong & Smith, 1997).

EcoP15I restriction enzyme must have a functional Mod subunit, i.e., capable of AdoMet binding, for DNA cleavage function (Bist *et al*., 2001). Earlier studies have also shown that EcoP1I restriction enzyme is essentially inactive in the absence of AdoMet (Janscak *et al*., 2001). This clearly indicates that AdoMet is a prerequisite for restriction function, whether prebound or added externally in the DNA cleavage reaction. AdoMet acts by bringing about necessary conformational changes in the enzyme.

A number of clear plaque mutants of phage P1 were generated in an attempt to elucidate the genes involved in the establishment of lysogeny. A cross-streak complementation test was employed to classify the mutants into four cistrons, termed *c*1, *c*2, *c*3, and *c*4. The phenotype of the *c*2 and *c*3 complementation groups of these clear plague mutants was elucidated (Rosner, 1973). Two of these, characterized as point mutations (Hümbelin *et al*., 1988), were shown to be modification deficient. The clear phenotype of *c*2 and *c*3 mutants of phage P1 were tentatively attributed to one of the following reasons: (1) that the gene products of these two cistrons were involved in controlling several functions, including lysogeny; (2) that the mutants were possibly modification deficient but were proficient in restriction (Rosner, 1973). The *c*2-134 mutant EcoP1 enzyme was not restriction proficient, while the *c*2-440 mutant enzyme was partially restriction proficient. The inability or partial ability of these enzymes to cleave DNA can be attributed to the fact that they are either unable to bind AdoMet or that they bind very weakly. Additionally, the clear plaque phenotype of the *c*2 mutants could probably be due to loss of many other functions and not because of their ability to self restrict. The *c*2-440 enzyme, which was able to bind AdoMet, albeit weakly, could be purified in large amounts and was stable, while the *c*2-134 mutant enzyme, which was unable to bind AdoMet, was relatively unstable. Complementation studies clearly indicated that while the presence of the mutant mod genes of the *c*2-134 or *c*2-440 phages resulted in efficiency of plating (e.o.p.) values corresponding to r^-m^- phenotype, introduction of the wild-type mod allele in this background resulted in e.o.p values corresponding to r^+m^+ phenotype (Krishnamurthy, 1996; Bist *et al*., 2001). The *c*2-134 mutant restriction enzyme purified from a mod^+ background and checked for restriction *in vitro* was found to restrict as well as modify the pUC18 DNA in the presence of AdoMet. This can be attributed to the *in vivo* reconstitution of wild-type Res subunit and wild-type Mod subunit to form an active restriction endonuclease. These observations indicate that the AdoMet-bound Mod subunit is somehow responsible for the stable expression of these enzymes. These observations are in agreement with earlier findings, that the *res* gene of either R.EcoP1I or R.EcoP15I cannot be expressed alone, and that a successful expression of the *res* gene was possible only when the *mod* gene was coexpressed (Krishnamurthy, 1996). It has been shown that the Mod subunit stabilizes the Res protein during expression (Redaschi & Bickle, 1996b).

AdoMet acts as the methyl group donor in most biological methylation reactions. However it does not act in this fashion in the cleavage of unmodified DNA by the type III R–M enzymes. It is clear from the discussion described above that AdoMet is required for specific DNA cleavage by type III R–M enzymes. As in the case of type I R–M enzymes, AdoMet may be required in the activation of the enzyme, which helps it to form a recognition complex. It has been proposed for the type I R–M enzymes that the binding of the cofactor triggers a slow transition of the enzyme to an activated species and, once activated, the enzyme cleaves double-stranded DNA. A similar scenario can be imagined for the type III R–M enzymes. It has been demonstrated that such conformational changes do occur upon cofactor binding to the enzyme (Bist *et al*., 2001). The studies with the type III enzymes provide strong evidence for the specific requirement of AdoMet for DNA cleavage function and as a positive allosteric effector of the DNA cleavage reaction.

It is interesting to note that the restriction enzymes that require AdoMet for DNA cleavage function are made up of different subunits, which either functionally cooperate (Type I and III) or are fused together (Type II B and G). This is in contrast to the enzymes that belong to the other type II families, such as the type II A, C, E, F, M, P, and S, which do not require AdoMet for their restriction function

and are homodimeric in function. The association of the endonuclease domains with the MTase domains led to the requirement of AdoMet in DNA cleavage (Murray, 2000; Bist *et al*., 2001; Kong & Smith, 1997).

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