2638–2643 *Nucleic Acids Research, 1999, Vol. 27, No. 13*

Single amino acid substitutions in the HsdR subunit of the type IB restriction enzyme *Eco*AI uncouple the DNA translocation and DNA cleavage activities of the enzyme

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Received March 29, 1999; Revised and Accepted May 18, 1999

ABSTRACT

Type I restriction enzymes bind to specific DNA sequences but subsequently translocate non-specific DNA past the complex in a reaction coupled to ATP hydrolysis and cleave DNA at any barrier that can halt the translocation process. The restriction subunit of these enzymes, HsdR, contains a cluster of seven amino acid sequence motifs typical of helicase superfamily II, that are believed to be relevant to the ATP-dependent DNA translocation. Alignment of all available HsdR sequences reveals an additional conserved region at the protein N-terminus with a consensus sequence reminiscent of the P-D...(D/E)-X-K catalytic motif of many type II restriction enzymes. To investigate the role of these conserved residues, we have produced mutants of the type IB restriction enzyme *Eco*AI. We have found that single alanine substitutions at Asp-61, Glu-76 and Lys-78 residues of the HsdR subunit abolished the enzyme's restriction activity but had no effect on its ATPase and DNA translocation activities, suggesting that these residues are part of the active site for DNA cleavage.

INTRODUCTION

Restriction enzymes are components of bacterial restriction-modification (R-M) systems that serve to protect the cells against bacteriophage infection, because the incoming foreign DNA is endonucleotically cleaved by the restriction enzyme if it contains the enzyme's recognition sequence. The cellular DNA is protected from cleavage by a specific methylation of the recognition sequences, which is introduced by the methyltransferase activity of the R-M system. R-M systems can be divided into several different types according to subunit composition and cofactor requirements (reviewed in 1,2).

In type I R-M systems, a multisubunit complex exhibits both restriction endonuclease and DNA methyltransferase activities. For the endonucleolytic activity, the enzyme complex requires S-adenosylmethionine (AdoMet), Mg^{2+} and ATP. In the presence of these cofactors, the enzyme binds to its recognition sequence but subsequently makes additional contacts with sequences near the recognition site and translocates DNA towards itself in a reaction accompanied by ATP hydrolysis. DNA cleavage occurs at non-specific sites that can be located far from the recognition sequence (reviewed in 3,4). The enzyme can translocate and cleave DNA on either side of its recognition sequence (5–7). DNA cleavage is triggered when the enzyme encounters a barrier that can cause a halt or pause in the translocation process (7). This can happen, for example, when two translocating enzyme molecules collide (5,6) or when a single enzyme molecule translocating a circular substrate from both directions has translocated the entire circle (7). Linear DNA with a single recognition site is a very poor substrate for type I restriction enzymes, since there is no barrier for tracking on such a molecule and the enzyme dissociates when it encounters the DNA end (6,8,9). One peculiarity of the type I restriction enzymes is that they do not turn over in the cleavage reaction, but they hydrolyze ATP long after DNA cleavage has stopped (3).

The type I R-M enzymes characterised to date are mostly from enterobacteria, although whole genome sequence data indicate that these enzymes are much more widely distributed (10,11). Based on subunit complementation, DNA hybridisation and antibody cross-reactivity experiments, the known type I R-M enzymes are grouped into four families, denoted IA–ID (12–14).

An active type I restriction enzyme is a pentameric complex of three different subunits, HsdR, HsdM and HsdS, which have a stoichiometry of $R_2M_2S_1$ (15,16). HsdM and HsdS can also form an independent DNA methyltransferase with a subunit stoichiometry of M_2S_1 (17,18). The HsdS subunit determines DNA specificity and it is composed of two separate DNA binding domains, each recognising one specific part of a non-palindromic sequence (19,20). The HsdM subunit contains the AdoMet binding site and the catalytic site for DNA methylation (21). The HsdR subunit is essential for restriction. It contains a set of seven amino acid sequence motifs typical for one superfamily of helicases and putative helicases, the so-called DEAD box proteins (22). These motifs, including an ATP-binding site motif, are clustered in the central region of the HsdR polypeptide (Fig. 1) and are thought to be associated with the ATP-dependent DNA translocation (23). Single amino acid changes in any of the DEAD box motifs of *Eco*KI, a type IA restriction enzyme, impair both ATPase and restriction endonuclease activities of the enzyme (24,25).

Amino acid sequence alignment of HsdR polypeptides from different families reveals the presence of an additional conserved region in the N-terminal part of HsdR (14). We have noted that the consensus sequence for this region (Fig. 1) is

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reminiscent of the P-D...(D/E)-X-K motif typical for the active site of many type II restriction enzymes (26). Here we describe properties of mutant forms of the type IB restriction endonuclease *Eco*AI containing alanine substitutions at the putative active site residues Asp-61, Glu-76 and Lys-78. We show that these residues are indeed important for restriction, but not for ATPase and DNA translocation activities of the enzyme. These data indicate that the N-terminal conserved region of HsdR contains active site residues for the DNA cleavage reaction.

MATERIALS AND METHODS

DNA manipulations

The origin repair method (27) was employed for site-directed mutagenesis. The original procedure was modified by usage of a DH5α *mutS* strain to enhance efficiency of mutagenesis (28). The mutagenesis of the *hsdR* gene was performed using the plasmid pJP41, a derivative of pGLamB (28) in which the *Xba*I–*Bam*HI fragment containing the *lamB* gene was replaced by the *Xba*I–*Bam*HI fragment of pJP22 carrying the *hsdR* gene. The *hsdR* gene in pJP41 is under control of the T7 transcriptional and translational signals. For *in vivo* functional assays, individual mutations were transferred from pJP41 derivatives to pFFP30 harboring all three *Eco*AI genes in the natural transcriptional organisation (29). This was achieved by replacing the *Nco*I–*Dra*III region in pFFP30 by the *Nco*I–*Dra*III fragments from the mutant derivatives of pJP41, resulting in plasmids named pFFP30D61A, pFFP30G69D, pFFP30E76A and pFFP30K78A, respectively.

Phage infection assay

The cells were grown in LB medium containing, when required, ampicillin at a concentration of 200 μg/ml to select for appropriate plasmids expressing wild-type or mutant *Eco*AI genes. At an OD_{600} of ~1, the cells were infected with serial dilutions of bacteriophage λ*vir* and phage titre was determined as described previously (30).

Protein preparations

Wild-type and mutant HsdR subunits were over-produced in *Escherichia coli* BL21 from the appropriate pJP41 derivative (see above), and purified to apparent homogeneity as described previously (31). HsdM and HsdS subunits of *Eco*AI were also produced separately and purified as described (31). The wild-type and mutant *Eco*AI endonucleases were reconstituted by mixing HsdR, HsdM and HsdS subunits in a ratio of 6:2:1. At this subunit ratio, the wild-type endonuclease reached the maximal activity when incubated with equimolar amounts of a circular DNA substrate containing one *Eco*AI recognition site (31). The excess of HsdR was presumably required due to the weak association of the two HsdR subunits with the methylase observed during the endonuclease purification (32). *Eco*KI endonuclease was produced from pVMC3 and purified as described (33).

DNA cleavage assay

All DNA cleavage reactions were performed at 37°C in buffer C [50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaCl, 1 mM dithiothreitol, 0.2 mM AdoMet, 5 mM ATP]. Reactions were started by the addition of ATP and stopped by the addition of 1 vol of 0.1 M EDTA (pH 8). Plasmids pJP25 (one *Eco*AI site) and pDRM.1R (no *Eco*AI site) were used as DNA substrates (31,34). If required, plasmid pJP25 was cut with *Alw*NI to produce a linear substrate. DNA and enzyme concentrations used are indicated in the figure legends. Reaction products were analyzed on agarose gels run in 0.5× TBE buffer. DNA was visualised by ethidium bromide staining. If required, agarose gel images were digitised and quantified using NIH Image 1.61 software.

ATPase assay

The ATPase activity of *Eco*AI mutants was measured by a colorimetric estimation of the inorganic phosphate released by ATP hydrolysis as described previously (34). Reaction conditions were the same as for the DNA cleavage assay.

RESULTS

The N-terminal conserved region of type I HsdR subunits contains an amino acid sequence motif reminiscent of the catalytic motif of type II restriction enzymes

Several short conserved regions have been identified by alignments of HsdR polypeptides from different type I restriction enzyme families (14,23; Fig. 1). These include the seven DEAD-box motifs, clustered in the central part of each polypeptide, whose relevance for both restriction and ATPase activities was demonstrated by mutational analysis of *Eco*KI (24,25) and a region that precedes the DEAD-box motifs, which was named region X (14). After including a number of HsdR homologues from recently sequenced eubacterial and archaeal genomes for sequence alignment using the Clustal X program (35), we have noted that the resulting consensus sequence for the region X (Fig. 1B) is reminiscent of the P-D...(D/E)-X-K catalytic motif of type II restriction enzymes such as *Eco*RI or *Eco*RV (26). The proline residue was not conserved in all HsdR subunits (Fig. 1B). However, proline is also absent in the catalytic motif of *Pvu*II (36) and it is dispensable in the catalytic motifs of several other type II endonucleases (37,38), which indicates that this residue is not required for catalysis. In almost all HsdR sequences, the two acidic residues of the motif are spaced by 13 amino acids except for the HsdR subunits of *Eco*AI and *Eco*EI (IB family) in which the spacer contains 14 amino acids. Similarly, the distance between the catalytic acidic residues in the primary sequence of type II enzymes is (with a few exceptions) in the range from 9 to 19 amino acids (39). In all HsdR polypeptides, the spacer contains additional conserved residues such as Gly and is highly hydrophobic (Fig. 1B). In the following text the putative DNA cleavage motif of type I restriction enzymes will be indicated as D-X_{13-14}-E-X-K.

Site-directed mutagenesis of *Eco*AI HsdR

To investigate the functional importance of the D-X_{13-14}-E-X-K sequence motif, we have produced mutants of the type IB restriction endonuclease *Eco*AI. The putative catalytic residues of *Eco*AI HsdR Asp-61, Glu-76 and Lys-78 (Fig. 1B) were individually substituted by an alanine. The conserved Gly-69 in the spacer between the acidic residues of the motif was also subjected to mutagenesis and it was replaced with an aspartic acid.

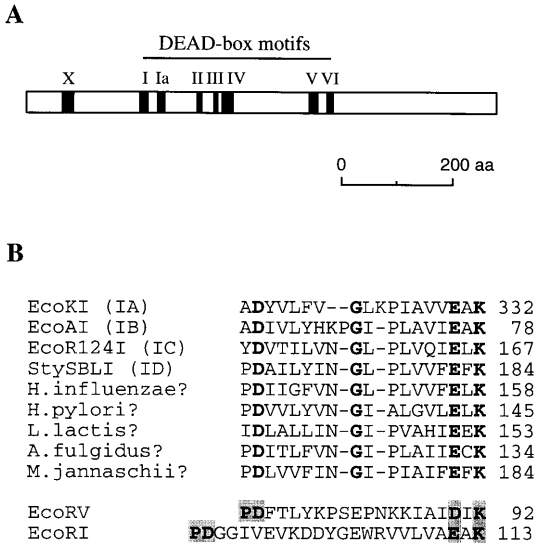

Figure 1. Conserved regions in the HsdR polypeptides of type I restriction enzymes. (**A**) A diagram of the HsdR subunit of the type IB restriction enzyme *Eco*AI showing the location of the conserved amino acid sequence regions as defined previously (14,23). Based on mutational analysis data, the conserved sequence originally named region Y was reclassified to be the DEAD-box motif IV (25). The original motif IV is not shown. (**B**) An alignment of amino acid sequences of several known and putative HsdR polypeptides around the N-terminal conserved region X. The number at the end of each line indicates the position for the last amino acid in the corresponding sequences. Conserved amino acids are indicated in bold. The regions of the type II endonucleases *Eco*RI and *Eco*RV containing the P-D...(D/E)-X-K catalytic motif are also shown. The elements of the motif are in grey boxes. The protein identification numbers used by the National Center for Biotechnology Information are as follows: *Eco*KI, 730887; *Eco*AI, 304894; *Eco*R124I, 78918; *Sty*SBLI, 1679868; *Haemophilus influenzae* Rd, 1574743; *Helicobacter pylori*, 2314575; *Lactococcus lactis*, 3057061; *Methanococcus jannaschii*, 2129337; *Eco*RI, 135227; *Eco*RI, 135230.

Restriction phenotype of mutants

The restriction phenotype of mutant endonucleases was determined by testing the ability of cells expressing these mutants to restrict the growth of unmodified bacteriophage λ. For this assay, individual mutations were transferred to the plasmid pFFP30 that contains all three *Eco*AI *hsd* genes in the natural transcriptional organisation (Materials and Methods). The resulting plasmids with mutant *hsdR* genes were transformed to DH5α and efficiency of plating (e.o.p.) of λ phage (e.o.p.; ratio of the phage titre on tested host to the titre on non-restricting host) on these strains was determined (Table 1). The data from this experiment indicated that the D61A, E76A and K78A mutants had greatly impaired restriction activity *in vivo*. The G69D mutant had a wild-type restriction phenotype, indicating that the conserved Gly residue is not important for this function.

In vitro DNA cleavage and ATPase activities of mutant enzymes

For *in vitro* analysis, the mutant HsdR subunits, except for G69D, were separately overproduced and purified as described in the Materials and Methods and mixed *in vitro* with purified HsdM and HsdS subunits to reconstitute the endonuclease. As a substrate for DNA cleavage *in vitro*, the plasmid pJP25 containing a single site for *Eco*AI was used. In agreement with the *in vivo* phenotypes, none of the mutant enzymes was able to cleave the plasmid DNA to its linear form even when present in a large excess over DNA (Fig. 2). Interestingly, the K78A mutant showed a significant nicking activity that was dependent on the presence of the *Eco*AI recognition site in the substrate (Fig. 2). The rate of the pJP25 DNA nicking by the K78A mutant was much lower than the rate of double-strand cleavage of this DNA by the wild-type enzyme (Fig. 3). The E76A mutant exhibited very little site-specific nicking activity and the D61A mutant did not show any nicking activity at all. None of the mutants exhibited any DNA cleavage activity on a linear DNA substrate containing two *Eco*AI recognition sites while the wild-type enzyme efficiently cleaved this substrate at random positions over the region between the recognition sites

Table 1. *In vivo* restriction of λ_{vir} by *Eco*A mutants

Plasmid	e.o.p.	(SD)
pFFP30	2.33×10^{-3}	($\pm 0.45 \times 10^{-3}$)
pFFP30D61A	0.47	(± 0.11)
pFFP30G69D	1.87×10^{-3}	($\pm 0.18 \times 10^{-3}$)
pFFP30E76A	0.49	(± 0.14)
pFFP30K78A	0.65	(± 0.11)

The strain DH5α (restriction minus) was used as the host for the plasmids expressing the wild-type (pFFP30) or mutant *Eco*A genes. Single amino acid substitutions in the HsdR subunit are indicated in the names of pFFP30 derivatives. The values of e.o.p. are averages of four independent measurements. The standard deviations (SD) are shown in brackets.

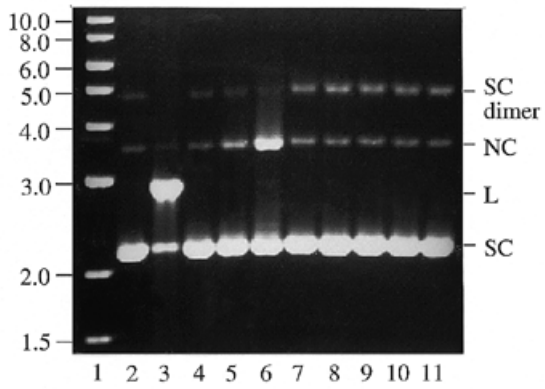

Figure 2. DNA cleavage assay of *Eco*AI mutants. The supercoiled form of the plasmid pJP25 (a single *Eco*AI site) was used as DNA substrate. Plasmid pDRM.1R (no *Eco*AI site) served as non-specific DNA for control reactions. 20 nM DNA was incubated with 80 nM wild-type or mutant *Eco*AI enzymes in buffer C at 37°C for 10 min. Reactions were stopped by the addition of 1 vol of 0.1 M EDTA (pH 8) and analysed on a 0.9% agarose gel run in 0.5× TBE buffer at 50 V for 5 h. DNA was visualised by ethidium bromide staining. Lane 1, DNA size markers; lanes 2–6 contain pJP25 incubated with no enzyme, wild-type, D61A, E76A end K78A, respectively; lanes 7–11 contain pDRM.1R incubated with no enzyme, wild-type, D61A, E76A and K78A, respectively. Positions of supercoiled (SC) linear (L), nicked (NC) and supercoiled dimeric (SC dimer) forms of plasmid DNA are indicated on the right of the gel.

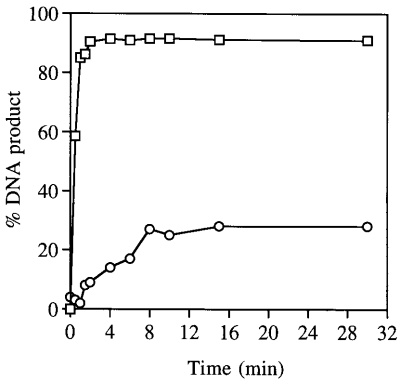

Figure 3. Rate of plasmid DNA cleavage by wild-type *Eco*AI endonuclease compared to the rate of DNA nicking by *Eco*AI$_{K78A}$ mutant. Cleavage reactions were carried out in buffer C at 37°C and contained 20 nM pJP25 DNA and 80 nM enzyme. Aliquots removed during the reaction were analysed on a 0.9% agarose gel run in 0.5× TBE buffer at 3 V/cm for 5 h. DNA was visualised by ethidium bromide staining. The gel was quantified as described in Materials and Methods and relative intensity of the linear DNA bands for wild-type enzyme (square) and nicked circular DNA bands for *Eco*AI$_{K78A}$ mutant (circle) in each time point was calculated as a percentage of total DNA per lane.

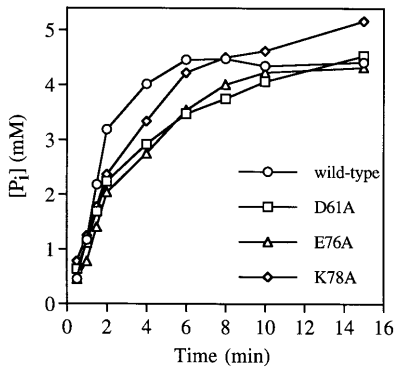

Figure 4. Time course of ATP hydrolysis by *Eco*AI mutants. 20 nM pJP25 DNA (a single *Eco*AI site) was incubated with 80 nM enzyme in buffer C at 37°C. During the incubation, 10 µl aliquots were removed and the reaction was stopped by the addition of 1 vol of 0.1 mM EDTA (pH 8). The concentration of inorganic phosphate (P_i) released by ATP hydrolysis was measured as described in Materials and Methods.

(not shown). Despite the absence of double-strand DNA cleavage activity, all three mutants had a similar ATPase activity to the wild-type endonuclease, indicating that the mutations did not cause a gross change in enzyme structure and that DNA cleavage is not a prerequisite for ATP hydrolysis (Fig. 4). Thus, the *in vitro* analysis of the *Eco*AI mutants suggests that all three charged HsdR residues of the D-X$_{13-14}$-E-X-K motif participate in the DNA cleavage reaction.

DNA translocation by *Eco*AI mutants

The ability of the *Eco*AI mutants to hydrolyse ATP suggested that these mutants could still translocate DNA. To investigate the DNA translocation activity of the mutant enzymes, we have examined their ability to promote linear DNA cleavage

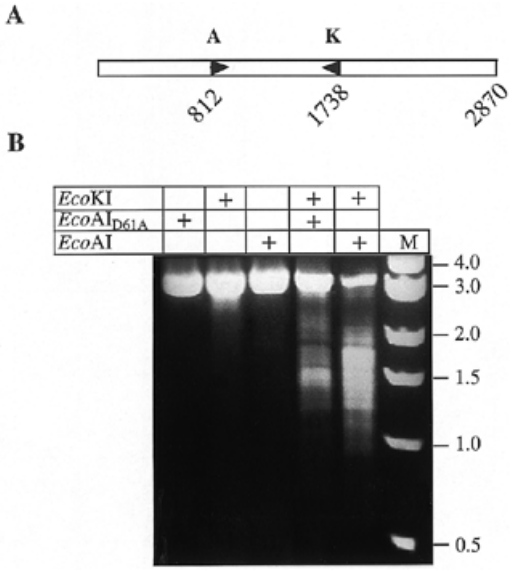

Figure 5. Effect of the *Eco*AI$_{D61A}$ mutant on linear DNA cleavage by the *Eco*KI endonuclease. (**A**) Diagram of DNA substrate (2870 bp) containing one *Eco*KI (K) site and one *Eco*AI (A) site that was prepared by *Alw*NI (unique site) cleavage of pJP25. DNA is represented as an open rectangle. The arrowheads indicate the orientation of the asymmetric recognition sites (tail-to-tail). The numbers show the position of the first base pair of the recognition sites. (**B**) Restriction assay. 13.25 nM DNA was incubated with 90 nM *Eco*KI and 80 nM *Eco*AI$_{D61A}$ individually or in combination in buffer C at 37°C for 8 min. In a control experiment, wild-type *Eco*AI endonuclease was used instead of *Eco*AI$_{D61A}$. Reactions were stopped by addition of 1 vol of 0.1 M EDTA and analysed on a 1% agarose gel run in 0.5× TBE buffer at 80 V for 2 h. DNA was visualised by ethidium bromide staining. Presence of enzymes in individual reactions is indicated above each lane by (+). Lane M contains DNA size markers.

by the type IA restriction enzyme *Eco*KI within the region between *Eco*KI and *Eco*AI recognition sites, by serving as molecular blocks to *Eco*KI translocation. This assay is based on our observation that type I restriction enzymes have the potential to cleave DNA at physical translocation barriers such as Holliday junctions, suggesting that DNA translocation blockage is the only requirement for DNA cleavage to occur (7). For this experiment, the pJP25 plasmid DNA was linearised by *Alw*NI to produce a 2.9 kb linear substrate with one *Eco*AI site and one *Eco*KI site separated by 0.9 kb (Fig. 5A). Assuming that the collision of *Eco*KI with the *Eco*AI mutants occurs at random positions between the recognition sites, the cleavage of this substrate would generate pairs of fragments with sizes ranging from ~0.8 to 2.0 kb. As expected, the incubation of the DNA substrate with a large excess of *Eco*KI for 8 min did not lead to significant DNA cleavage (Fig. 5B), consistent with the requirement for the presence of two enzyme recognition sites for efficient linear DNA cleavage (6,8,9). However, when the reconstituted *Eco*AI$_{D61A}$ mutant was added together with *Eco*KI, extensive DNA cleavage occurred generating multiple products which appeared as a DNA smear on EtBr-stained gel, consistent with random cleavage (Fig. 5B). As judged from the electrophoretic mobilities of the cleavage products relative to DNA size markers, the cleavage events occurred predominately within the region between the *Eco*KI and *Eco*AI recognition sites, with some preference for a very short area located

approximately in the middle of the DNA molecule (~200 bp from the *Eco*KI site). Identical cleavage profiles were also obtained with the E76A and K78A mutants when combined with *Eco*KI (not shown). In a control experiment, *Eco*KI was combined with the wild-type *Eco*AI endonuclease. As observed previously (7), this enzyme combination led to efficient cleavage at random positions over the region between the *Eco*AI and *Eco*KI sites, with some preference for the area starting approximately half way between the sites and ending at the *Eco*KI site (Fig. 5). One can notice that DNA cleavage resulting from the cooperation between *Eco*KI and *Eco*AI$_{D61A}$ mutant was less efficient as compared to the situation when the wild-type *Eco*AI endonuclease was present instead of the mutant (Fig. 5). This may reflect the fact that in the later case DNA cleavage can be catalysed by either enzyme while in the former case only *Eco*KI is active for cleavage.

Thus, the fact that the *Eco*AI mutants could promote DNA cleavage by *Eco*KI far from the *Eco*AI recognition sequence clearly demonstrates that these mutants are capable of translocating DNA.

DISCUSSION

Conservation of an amino acid sequence motif reminiscent of the P-D…(D/E)-X-K catalytic motif of many type II restriction enzymes in the N-terminal region of all known and putative HsdR subunits (Fig. 1) suggested that this motif may be a candidate for the endonucleolytic active site of type I restriction enzymes. In the active centre of the type II endonucleases, the two acidic amino acid residues serve to coordinate a Mg^{2+} ion that is the cofactor for the phosphodiester bond hydrolysis. The lysine residue is thought to stabilise the doubly charged pentavalent transition state (26). We have made single alanine substitutions in the putative active site motif of the HsdR subunit of the type IB restriction enzyme *Eco*AI, formed by Asp-61, Glu-76 and Lys-78 residues. These amino acid substitutions resulted in enzymes that exhibited normal ATPase activity and the ability to translocate DNA, but they failed to cleave DNA. Hence, all the three mutations have the properties expected for the mutations at the active site residues for DNA cleavage; they uncouple ATP-dependent DNA translocation and restriction activities of the enzyme. It is quite likely that the homologous motifs in other type I restriction enzymes also form the endonucleolytic active site since they share common features with the active site residues of *Eco*AI: (i) they are located in the same part of the HsdR polypeptide and (ii) the sequences between the two acidic residues are homologous (Fig. 1).

The K78A mutant of *Eco*AI exhibited a significant site-specific nicking activity (Figs 2 and 3). Since the D61A and E76A mutants had no or very little nicking activity (Fig. 2), the ability of the K78A mutant to nick plasmid DNA substrate may reflect residual activity of the mutated cleavage centre rather than the presence of an additional active site for DNA cleavage. Similarly, nicking activity was observed with *Eco*RV catalytic mutants (40). In addition, an alanine substitution at the lysine residue of *Eco*RV did not completely eliminate double strand cleavage activity while alanine mutants at either acidic residue were completely inactive in double-strand cleavage (40).

In all HsdR polypeptides, the putative active site for DNA cleavage is located outside of the helicase-like domain (Fig. 1). This may suggest a modular protein structure, with the latter domain involved in ATP-dependent DNA translocation. Some support for this assumption comes from our finding that alanine substitutions at the endonucleolytic active site of the HsdR subunit of *Eco*AI effectively uncoupled the DNA translocation and DNA cleavage activities of the enzyme. A protein structure composed of independent nuclease and helicase domains was described for the RecB subunit of the *E.coli* RecBCD enzyme (41). Mutations in the DEAD-box motifs of *Eco*KI impaired both ATPase and restriction activity (25). It is possible that interactions of the helicase-like domain with ATP and DNA are required for an activation of the cleavage domain, perhaps by positioning this domain in proximity to the DNA substrate. All DEAD-box mutants exhibited some site-specific nicking activity (25), which may reflect a residual activity of the cleavage domain.

The mutants produced in this work could be useful in studying the mechanism of DNA translocation by type I restriction enzymes, since the experiments with the wild-type enzyme are hampered by rapid cleavage of DNA substrates.

ACKNOWLEDGEMENTS

We are grateful to Daniel Panne for comments on the manuscript. This work was supported by grants from the Swiss National Science Foundation.

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