Domain Organization and Functional Analysis of Type IIS Restriction Endonuclease *Eco*31I[†]

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ABSTRACT: Type IIS restriction endonuclease *Eco*31I harbors a single HNH active site and cleaves both DNA strands close to its recognition sequence, 5′-GGTCTC(1/5). A two-domain organization of *Eco*31I was determined by limited proteolysis. Analysis of proteolytic fragments revealed that the N-terminal domain of *Eco*31I is responsible for the specific DNA binding, while the C-terminal domain contains the HNH nuclease-like active site. Gel-shift and gel-filtration experiments revealed that a monomer of the N-terminal domain of *Eco*31I is able to bind a single copy of cognate DNA. However, in contrast to other studied type IIS enzymes, the isolated catalytic domain of *Eco*31I was inactive. Steady-state and transient kinetic analysis of *Eco*31I reactions was inconsistent with dimerization of *Eco*31I on DNA. Thus, we propose that *Eco*31I interacts with individual copies of its recognition sequence in its monomeric form and presumably remains a monomer as it cleaves both strands of double-stranded DNA. The domain organization and reaction mechanism established for *Eco*31I should be common for a group of evolutionary related type IIS restriction endonucleases *Alw*26I, *Bsa*I, *Bsm*AI, *Bsm*BI and *Esp*3I that recognize DNA sequences bearing the common pentanucleotide 5′-GTCTC.

The type II restriction endonucleases (REases[1]) recognize short DNA sequences and cut phosphodiester bonds at fixed positions within or close to their recognition site. The value of REases for DNA manipulations has prompted extensive searches for new enzymes of this sort, and over 3700 such enzymes have been identified. Despite a similar function, REases are an extremely versatile group of proteins. They can be classified according to two types of criteria based either on the type of the catalytic center or on the structural and functional properties of the enzyme.

Currently, five types of catalytic centers are identified in REases: PD-(E/D)XK, PLD, GIY-YIG, HNH and "half-pipe". Among these families, the enzymes of PD-(E/D)XK family are best characterized both structurally and with respect to the mechanism of DNA cleavage (*1*). The PD-(E/D)XK enzymes are Mg^{2+}-dependent endonucleases that use acidic residues to coordinate Mg^{2+}-ions, which are a necessary cofactor for DNA hydrolysis. In contrast to the Mg^{2+}-dependent endonucleases of PD-(E/D)XK family, the phospholipase D (PLD) superfamily enzymes *Bfi*I (*2*) and *Bmr*I (*3*) do not require any metal ions for catalysis. *Bfi*I uses a single active site to cut sequentially both DNA strands and employs a covalent DNA intermediate in catalysis (*4, 5*). Comparative amino acid analysis and mutational data show that REases *Kpn*I (*6*), *Mnl*I (*7*) and *Eco*31I (*8*) are members of the HNH nuclease family, which also includes some homing endonucleases and bacterial colicins. Catalytic activity of HNH REases is supported by a wide range of divalent metal ions, including Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} (*9*). The GIY-YIG family REases *Eco*29kI and *Cfr*42I were also identified by bioinformatic methods and mutational analysis (*10*). Recent biochemical studies of *Cfr*42I revealed that GIY-YIG REases, similarly to HNH enzymes, are also promiscuous regarding their divalent metal-ion cofactor requirement: their catalytic function is supported by Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} (*11*). REase *Pab*I shows a novel nuclease fold and is a founding member of yet another nuclease family named "half-pipe" (*12*).

Alternative classification of REases is based on their structural−functional properties. Based on the symmetry of the recognition sequences, oligomeric structure, DNA cleavage positions and cofactor requirements, REases are divided into more than ten subtypes (*13*). The orthodox restriction enzymes are homodimeric single-domain proteins. They symmetrically bind palindromic recognition sites, so that one active site from the dimer is positioned against one DNA strand and likewise the second active site on the other strand. Independent reactions in each active site then generate the double-strand break. However, several REases, including *Msp*I, *Bcn*I and *Mva*I, bind their palindromic sequences in

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[1] Abbreviations: REase, restriction endonuclease; CTD, C-terminal domain; NTD, N-terminal domain.

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the monomeric form and presumably need dissociation and rebinding in the opposite orientation to achieve cleavage of two DNA strands (*14–17*). Most orthodox REases employ the PD-(E/D)XK active sites, though other types of active sites are also reported: *Pab*I, *Kpn*I and *Eco*29kI belong to the "half-pipe", HNH and GIY-YIG nuclease families, respectively.

Type IIF REases are homotetramers in solution and are arranged as dimers of dimeric orthodox REases (*18–20*). These enzymes simultaneously bind two DNA molecules and cleave four phosphodiester bonds in a concerted reaction generating two double-strand breaks. Most type IIF REases also belong to the PD-(E/D)XK family, though homotetrameric enzyme *Cfr*42I belongs to the GIY-YIG nucleases (*11*).

Type IIE and type IIS REases are characterized by domain structure. IIE enzymes *Eco*RII and *Nae*I resemble orthodox PD-(E/D)XK enzymes that contain additional effector domains (*21, 22*). These enzymes require simultaneous binding of two or even three copies of the recognition sequence to both the catalytic and effector domains for catalytic activity (*23*). Unlike the IIF enzymes, type IIE REases hydrolyze only one of the bound DNA molecules.

The most versatile and least understood group of REases is named type IIS. These nucleases recognize asymmetric DNA sequences and cleave DNA outside of the recognition site (*13*). The characterized type IIS enzymes are composed of separate DNA recognition and catalytic domains, but contrary to the type IIE enzymes, only the DNA recognition domain is able to bind cognate DNA. To date, monomeric (*Fok*I (*24*), *Mva*1269I (*25*)), homodimeric (*Bfi*I (*26*)) and homotetrameric (*Bsp*MI (*27*)) type IIS REases were identified. Their catalytic domains belong to PD-(E/D)XK (*Fok*I (*24*), *Mva*1269I (*25*)), HNH (*Mnl*I (*7*), *Eco*31I (*8*)) and PLD (*Bfi*I (*28*)) families. Biochemical analysis of type IIS enzymes revealed an extreme diversity of reaction mechanisms and strategies employed to cleave double-stranded DNA (*5, 25, 29*).

However, currently we have little information about the domain organization and reaction mechanism of type IIS REases that cleave DNA strands close to their recognition sequences. Therefore, we determined domain organization and possible reaction mechanism of the type IIS REase *Eco*31I that cleaves DNA 1/5 nucleotides downstream of the 5′-GGTCTC recognition site and harbors a single HNH nuclease-like active site (*8, 30, 31*). We show here that *Eco*31I consists of the N-terminal DNA binding and C-terminal catalytic domains and presumably remains a monomer as it binds and cleaves double-stranded DNA.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Reagents. The *Escherichia coli* strains ER2267 (New England Biolabs) and HMS174 (DE3) (Novagen) were used as hosts for expression of *Eco*31I (GenBank/EMBL/DDBJ accession no. AAM09638) and its domains. *E. coli* cells were grown in LB broth or on LB agar supplemented with appropriate antibiotics (*32*). REases, DNA modification enzymes, molecular biology kits, DNA and protein molecular weight markers were from Fermentas. [γ-^{33}P] ATP was from Amersham. The proteases were purchased from Roche. Porablot PVDF membrane for protein blotting was from Macherey-Nagel. Oligonucleotides used for gel-mobility shift assay and PCR were synthesized by MWG-Biotech and Metabion.

DNA Manipulations. Plasmid DNA isolation, DNA restriction, agarose gel electrophoresis, isolation of individual DNA fragments from agarose gels and subcloning of DNA fragments were carried out by standard procedures (*32*). Nucleotide sequences were generated using "Big-Dye" terminator chemistry and data collected on Genetic Analyzer 3130*xl* (Applied Biosystems).

Oligonucleotides. The single stranded oligonucleotide (5′-TTTCTCTTAGGTTACGGTCTCTACTGCT-TGTCGTCAGGCT) was annealed to the complementary one (5′-TTTAGCCTGACGACAAGCAGTAGAGACCG-TAACCTAAGAG) to give specific 37/43-DNA with protruding 3 nucleotide 5′-termini. *Eco*31I target sequence (underlined) was substituted by GGCCAC (top strand) to give nonspecific 37/43-DNA. The fluorescein-labeled oligoduplex 19/21-DNA-Fluo (5′-Fluo-TCGTGGGTCTCGCG-GTATCA/5′-TTGATACCGCGAGACCCACG) with protruding 1 nucleotide 5′-termini was used for gel-filtration experiments.

Construction of the Plasmids. Plasmid carrying two *Eco*31I target sequences, pEco31I-2, was constructed by cloning 311 bp *Hpy*8I-*Nsb*I fragment containing the *Eco*31I site from pUC19 into pUC19 precut with *Sma*I. The *Eco*31I targets are separated by ~1.5 kb and oriented in the same direction. The plasmids were isolated using standard alkaline lysis procedure (*32*) followed by purification by QIAquick PCR purification kit (Qiagen). 85% of isolated DNA was in the SC form, the remaining DNA contained random nicks.

The *eco31IR* gene fragments coding for N-terminal and C-terminal parts of Arg-C digested *Eco*31I were amplified using plasmid pΔ2-Eco31IR as template (*8*). The N-terminal part of *Eco*31I up to 251st coding residue of *eco31IR* gene and the C-terminal part beginning from the additional ATG codon upstream the 252nd coding residue of *eco31IR* gene were inserted into pET-21b(+) (Novagen) under control of T7lac promoter. The sequences of PCR products were verified by sequencing.

Protein Purification. The full-length *Eco*31I from the induced *E. coli* 2267 [pΔ2-Eco31I+p184-Eco31IM] strain was purified as described previously (*8*). *E. coli* HMS174 [pET21-Eco31I-N+p184-Eco31IM] and [pET21-Eco31I-C+p184-Eco31IM] strains were used for overexpression of *Eco*31I-N and *Eco*31I-C. Cells were grown at 37 °C with aeration in LB broth to OD_{600} of ~0.5. Expression of the genes was induced with 1 mM IPTG, and after 3 h at 37 °C the cells were harvested by centrifugation and stored at −20 °C. All further steps were carried out at 4 °C. Biomasses were thawed in buffer A (10 mM K-phosphate (pH 7.0), 1 mM EDTA, 7 mM 2-mercaptoethanol and 100 mM KCl). Then cells were sonicated and cell debris was removed by centrifugation. To remove nucleic acids, polyethyleneimine (pH 7.5) solution was gradually added to a final concentration of 1% from 10% stock solution and the precipitate was removed by centrifugation. Soluble proteins were fractionated by saturating the solution with $(NH_4)_2SO_4$. Proteins that precipitated at 40% − 60% saturation were dissolved in buffer A and dialyzed against the same buffer. Obtained dialysates were purified according to individual purification schemes. The dialysate containing *Eco*31I-N was sequentially

loaded on phosphocellulose P-11 (Whatman), Heparin-Sepharose, AH-Sepharose and Blue-Sepharose columns (Amersham-Pharmacia). The dialysate containing *Eco*31I-C was sequentially loaded on phosphocellulose P-11, Q-Sepharose, hydroxyapatite (Whatman), Blue-Sepharose, Bordo-Sepharose and AH-Sepharose columns. Eluted *Eco*31I-N and *Eco*31I-C fractions assayed by SDS−PAGE were pooled and dialyzed against storage buffer and stored at −20 °C.

The proteins obtained were >95% homogeneous as judged by SDS−PAGE analysis. Protein concentrations were determined spectrophotometrically by absorbance at 280 nm, using calculated extinction coefficients. The concentrations are given in terms of the monomeric form.

Proteolysis and Amino Acid Sequence Analysis. Purified *Eco*31I (50 μg) was incubated with different amounts of proteases at 25 °C in 200 μL of selected buffer: TAE for trypsin; 90 mM Tris-HCl (pH 7.6), 8.5 mM $CaCl_2$, 5 mM DTT, 0.5 mM EDTA for endoproteinase Arg-C and 25 mM ammonium-carbonate (pH 7.8) for endoproteinase Glu-C. At timed intervals, 20 μL aliquots of the reactions were stopped by precipitation with CCl_3COOH and Na-deoxycholate (*32*). After dissolving in 0.1 M NaOH, the reaction products were fractionated by SDS−PAGE. Gels were either stained with Coomassie brilliant blue R-250 or semidry blotted on PVDF membrane for protein sequencing. Molecular mass of observed polypeptides was estimated using calibration curve generated by standard proteins: β-galactosidase (166 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), REase *Bsp*98I (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa).

Polypeptide fragments were visualized as bands on PVDF membrane by staining with Coomassie brilliant blue R-250. Membranes were subjected to N-terminal amino acid sequencing at Max Planck Institute (Dortmund, Germany).

Effect of DNA Binding on Proteolysis of Eco31I. To assess the effect of DNA binding on proteolysis of *Eco*31I, 50 μg of *Eco*31I was incubated with trypsin at 25 °C in 200 μL of TAE buffer for 20 min in the presence of specific or nonspecific 37/43-DNA (*Eco*31I:DNA molar ratio 1:2). At timed intervals, 20 μL aliquots of the reaction mix were precipitated (see above) and the reaction products were fractionated by SDS−PAGE.

Gel-Mobility Shift Assay. 5′-Termini of single-stranded oligonucleotides were labeled with T4 polynucleotide kinase and [γ-^{33}P]ATP and annealed to unlabeled complementary one to give probe DNA. The radiolabeled specific or nonspecific 37/43-DNA (in final concentration 1 nM) was incubated with different amounts of selected protein in 33 mM Tris-acetate (pH 7.9), 66 mM K-acetate, 1 mM EDTA, 0.1 mg/mL BSA and 10% glycerol at 25 °C for 20 min in the total volume of 20 μL. After 20 min incubation the samples were loaded onto 8% polyacrylamide non-denaturing gels (29:1 acrylamide/bisacrylamide) and fractionated in TAE buffer (40 mM Tris-acetate, pH 8.0, 2 mM EDTA). Results were analyzed using Cyclone Storage Phosphor System with OptiQuant 3.0 software (Packard).

Gel-Filtration Assay. Gel-filtration of the purified proteins and their mixtures with DNA was performed at room temperature on the AKTA Explorer system using Superdex 200 HR column (Amersham-Pharmacia) pre-equilibrated with TAE buffer with 150 mM Na-acetate. The samples of proteins and DNAs were prepared in 200 μL of the same buffer. Elution from the column was monitored by measuring absorbance at 280 nm (detection of protein and DNA) and at 495 nm (detection of fluorescein-labeled DNA). The calibration curve was generated using Gel-filtration Calibration kit (Amersham-Pharmacia). The molecular masses of proteins and their complexes with DNA were calculated by interpolating their elution volumes onto the calibration curve.

Restriction Activity In Vitro Assay. The conditions for testing nuclease activity of *Eco*31I-N, *Eco*31I-C and equimolar mix of both fragments were as follows: 10 nM protein and 11.4 nM of intact pUC19 or linearized pUC19/*Pst*I were incubated in Five Buffer System (Fermentas) and KGB buffer (*33*) in total 50 μL volume for 18 h at 37 °C. The probe without any tested protein served as negative control. The reactions were stopped by adding 6× loading dye and SDS solution (Fermentas) and heating at 70 °C for 10 min. The reaction products were analyzed by agarose gel electrophoresis.

Cleavage of One-Site and Two-Site Plasmid Substrates by Eco31I. Multiple-turnover cleavage reactions were performed at 25 °C with 10 nM plasmid DNA (pUC19 or pEco31I-2) and *Eco*31I (0.1−0.8 nM) in 33 mM Tris-acetate (pH 7.9 at 25 °C), 66 mM K-acetate, 10 mM Mg-acetate and 0.1 mg/mL BSA. Aliquots were removed at timed intervals and quenched by adding 3× loading dye solution containing 75 mM EDTA and 0.3% SDS. The reaction products were analyzed by electrophoresis through agarose as described (*26*). Single-turnover experiments were performed by mixing equal volumes (16 μL each) of magnesium-acetate solution with the preincubated mixture of enzyme and plasmid DNA in a quench-flow device (KinTek Corporation). Final concentrations were 20−40 nM enzyme monomer, 5 nM plasmid DNA and 10 mM Mg^{2+} in a pH 7.9 reaction buffer mentioned above at 25 °C. The reactions were quenched with 6 M guanidinium chloride. DNA was precipitated and analyzed as described (*26*).

RESULTS

Limited Proteolysis of Eco31I. Protein peptide bonds accessible to proteases generally occur in extended linker regions or loops between domains (*34*). To determine the possible interdomain regions of *Eco*31I, we subjected the purified protein to limited proteolysis by three residue-specific endoproteinases Arg-C, Glu-C and trypsin. Each of the three digests was monitored by SDS−PAGE (Figure 1). In the beginning of the Arg-C reaction, two major *Eco*31I fragments of 38 kDa and 29 kDa were formed. The 38 kDa fragment was resistant to further Arg-C digestion, while the 29 kDa fragment was completely degraded after 90 min incubation (Figure 1A). Two major fragments of 35−38 kDa and 26−29 kDa were also generated upon proteolysis with Glu-C and trypsin (Figure 1B,C), suggesting that cleavage sites of all tested enzymes are located in the same *Eco*31I region. We determined the N-terminal sequences of selected proteolytic fragments obtained with Arg-C (38 kDa and 29 kDa) and Glu-C (36 kDa, 29 kDa and 27 kDa) (Table 1). The 38 kDa and the 36 kDa polypeptides start at residues 252T and 255N, respectively, and therefore originate from the C-terminal part of *Eco*31I. The smaller 29 kDa and 27 kDa fragments start at residues 1−6 and 23, respectively, and thus correspond to the N-terminal part of the enzyme (Table 1). The sum of apparent molecular masses of the

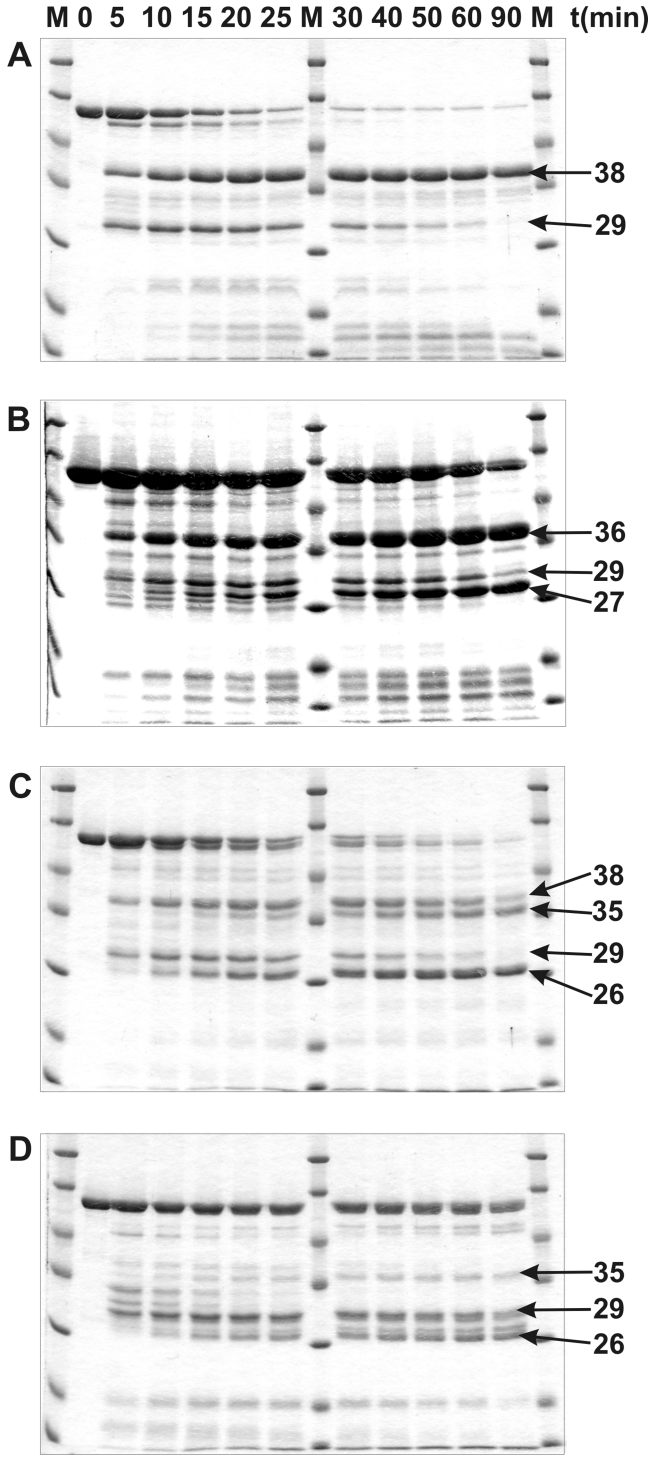

FIGURE 1: Limited proteolysis of *Eco*31I. SDS−polyacrylamide gels showing patterns of polypeptide fragments produced by limited *Eco*31I digestion with Arg-C (A), Glu-C (B) and trypsin (C) in the absence of DNA. Trypsin digestion of *Eco*31I in the presence of specific DNA (D). The time of digestion is indicated alongside the top of the lanes. M, protein molecular mass standard (see Experimental Procedures). The calculated molecular masses (kDa) of protease-resistant fragments are indicated alongside the gel images.

obtained N- and C-terminal fragments is close to the calculated molecular mass of *Eco*31I (67 kDa). Based on this analysis, we conclude that *Eco*31I consists of two major protease-resistant domains linked by a region susceptible to proteases.

Table 1: Major polypeptides produced by limited proteolysis of *Eco*31I

protease	molecular mass (kDa)	N-terminal sequence
Arg-C	29[a]	1-MKMGXIAVTP-20%
		4-GKIAVTPNND-70%
		6-IAVTPNNDXA-10%
	38	252-TKENLKSYVT
Glu-C	29[a]	1-MKMGXIAVTP-20%
		4-GKIAVTPNND-70%
		6-IAVTPNNDXA-10%
	27	23-NLATNIKK
	36	255-NLKSYVTD

[a] The 29 kDa protein bands were found to be mixtures of three fragments due to the ambiguous translation at the initiation position. "X" designates undetermined amino acid.

Effect of DNA Binding on Eco31I Proteolysis. DNA-induced conformational rearrangements alter the susceptibility of REases and other DNA-binding proteins to proteases (*9, 35–41*). To identify the regions of *Eco*31I that either interact with cognate DNA or undergo conformational rearrangements upon DNA binding, we compared the *Eco*31I trypsin digestion patterns in the presence and absence of DNA (Figure 1C,D).

*Eco*31I digestion with trypsin in the presence of specific 37/43-DNA did not alter the size of obtained fragments, but greatly attenuated digestion rate of the full-length *Eco*31I (Figure 1C,D). Furthermore, in the presence DNA, the 38 kDa and 35 kDa polypeptides corresponding to the C-terminal domain (CTD) of *Eco*31I were hardly detectable even in the beginning of the reaction. In contrast, conversion of the 29 kDa N-terminal domain (NTD) into the 26 kDa fragment was strongly retarded (Figure 1D), suggesting that NTD is involved in cognate DNA binding. Unlike the cognate oligoduplex, nonspecific DNA had no effect on either the *Eco*31I proteolysis pattern or the digestion rate (data not shown).

Analysis of Nuclease Activity of Protease-Resistant Domains. In order to characterize the individual NTD and CTD of *Eco*31I, we attempted to separate 38 kDa and the 29 kDa fragments obtained by Arg-C treatment using ion-exchange and gel-filtration chromatography. However, we succeeded to isolate only the 38 kDa CTD. To overcome this problem, we have cloned fragments of the *eco31IR* gene representing the NTD and CTD. Proteins *Eco*31I-N (NTD of *Eco*31I) and *Eco*31I-C (CTD of *Eco*31I) were overexpressed and purified as described in Experimental Procedures.

To test the nuclease activity of *Eco*31I domains, we carried out DNA cleavage experiments with both supercoiled and linear DNA substrates. Neither separately isolated *Eco*31I-N and *Eco*31I-C nor their equimolar mixture showed any detectable activity even after prolonged incubation in different reaction buffers (data not shown). The same was true for the CTD isolated after *Eco*31I proteolysis with Arg-C.

Bioinformatical and mutational analysis of *Eco*31I identified a single HNH nuclease-like active center $D_{311}H_{312}$-$(x)_{21}$-N_{334} that resides in the C-terminal domain of *Eco*31I (*8*). Undetectable nuclease activity of isolated CTD and its accelerated degradation in the presence of specific DNA suggest that, upon cognate DNA binding, the CTD of full-length *Eco*31I undergoes conformational rearrangements that presumably enable its catalytic function.

Identification of DNA-Binding Domain by Gel Mobility-Shift Assay. Gel mobility-shift assay was used to determine

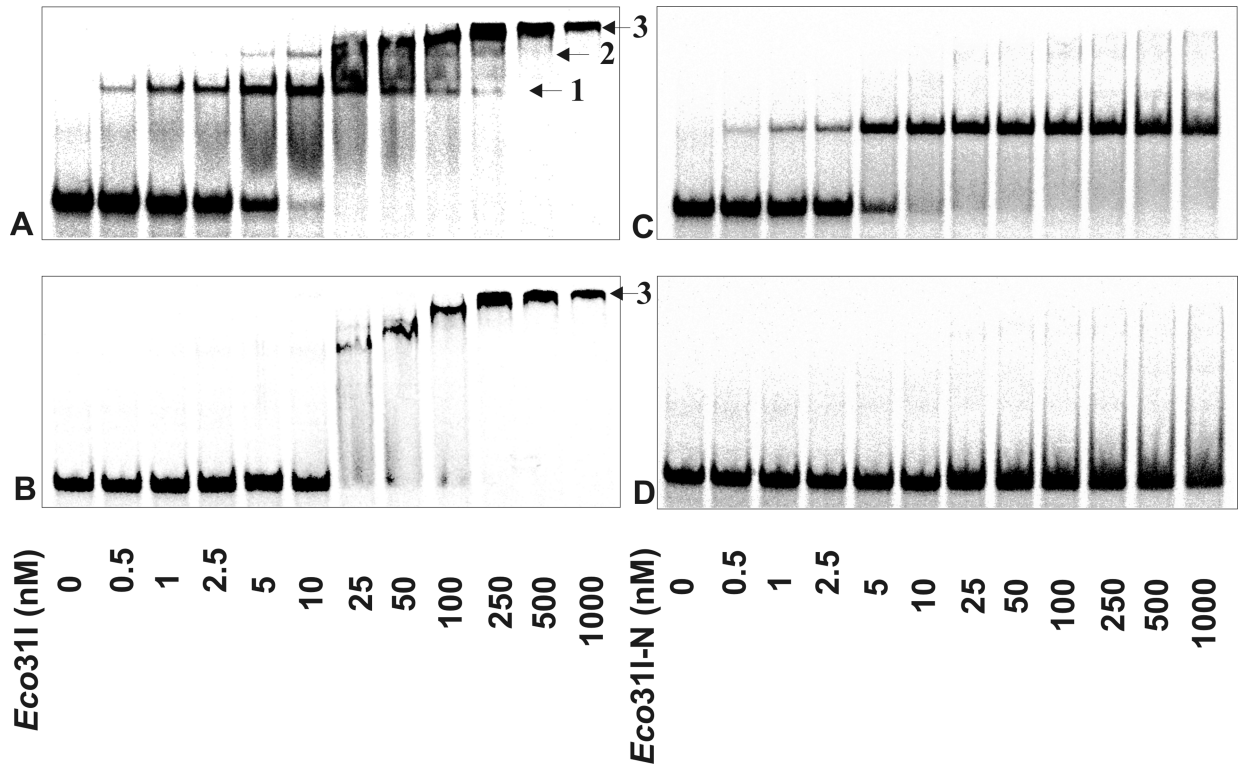

FIGURE 2: Gel mobility-shift analysis of full-length *Eco*31I and its N-terminal domain, *Eco*31I-N. Binding of *Eco*31I to the specific (A) and nonspecific (B) 37/43-DNA. Three types of *Eco*31I−DNA complexes formed with cognate DNA are indicated by arrows. Binding of *Eco*31I-N to the specific (C) and nonspecific (D) 37/43-DNA. In all experiments the concentration of DNA was 1 nM. The protein concentrations used in each reaction are shown below each lane.

DNA-binding characteristics of the full-length *Eco*31I, *Eco*31I-N and *Eco*31I-C. We used two DNA oligoduplexes: specific 37/43-DNA and nonspecific 37/43-DNA. The gel mobility-shift assay of *Eco*31I binding with the specific 37/43-DNA revealed a shifted DNA band already at the initial (0.5 nM) enzyme concentration (Figure 2A). The amount of the initial complex (complex 1) increased with increasing protein concentration in the range of 0.5−10 nM. The second shifted band of lower mobility (complex 2) appeared at enzyme concentration of 5 nM. Finally, at 25−1000 nM of enzyme, a smear of low mobility complexes that hardly entered the gel appeared (complex 3, Figure 2A). Similar studies of full-length *Eco*31I with nonspecific 37/43-DNA revealed shifted bands at 10−1000 nM of enzyme (Figure 2B).

Comparison of *Eco*31I interaction with specific and nonspecific DNAs suggests that shifted band observed only with the cognate DNA (complex 1) corresponds to the specific enzyme−DNA complex. In contrast, the low mobility smear (complex 3) observed with cognate DNA has an equivalent in the experiment with nonspecific oligonucleotide (Figure 2A,B) and thus corresponds to the nonspecific *Eco*31I−DNA complex with multiple enzyme molecules bound to DNA. However, we could not unambiguously assign the minor band of intermediate mobility (complex 2, Figure 2A) to either specific or nonspecific *Eco*31I−DNA complex. One possibility is that complex 2 is a cognate *Eco*31I−DNA complex of different stoichiometry than complex 1. Alternatively, complex 2 may represent the fastest-moving fraction of nonspecific complexes, as band 2 is formed at similar *Eco*31I concentrations (5−10 nM, Figure 2A) as the *Eco*31I complex with nonspecific DNA (10−25 nM, Figure 2B).

The gel mobility-shift assay of *Eco*31I-N with the specific 37/43-DNA revealed a single shifted DNA band across the whole range of protein concentrations (0.5−1000 nM, Figure 2C). The retarded DNA bands were not observed in the *Eco*31I-N binding experiments with nonspecific DNA (Figure 2D). No DNA-binding activity was detected in the gel mobility-shift experiments with *Eco*31I-C (data not shown).

Gel-Filtration Experiments. Previous gel-filtration studies suggested that *Eco*31I is a monomer in solution and, in the presence of specific 20/28-DNA, binds one DNA molecule forming the 1 + 1 complex. At elevated enzyme concentrations, *Eco*31I also forms complexes of higher molecular weight (*8*). To avoid nonspecific binding of *Eco*31I to DNA, we repeated the *Eco*31I gel-filtration experiments employing a shorter oligoduplex, 19/21-DNA-Fluo. To enable comparison of protein−DNA stoichiometry in different *Eco*31I−DNA complexes, the 19/21-DNA-Fluo duplex also carried a fluorescein label. Absorbance recording at 495 nm enabled monitoring of DNA concentration alone, and readings at 280 nm revealed the total concentration of both protein and DNA.

The 19/21-DNA-Fluo oligoduplex eluted at a volume that, relative to the marker proteins, yielded an apparent molecular mass of 30 kDa (Figure 3A). This value is more than two times higher than its actual molecular mass of 12.8 kDa. Observed discrepancy is due to the duplex having, on account of its cylindrical shape, a much higher frictional ratio than the spherically shaped marker proteins. Free *Eco*31I eluted at a volume that corresponds to an apparent molecular mass of 62 kDa, a close match to the calculated mass of *Eco*31I monomer (67 kDa). The 19/21-DNA-Fluo and *Eco*31I mixtures containing constant amount of DNA (0.5 μM) and varied amounts of *Eco*31I (1.0−2.0 μM) yielded peaks

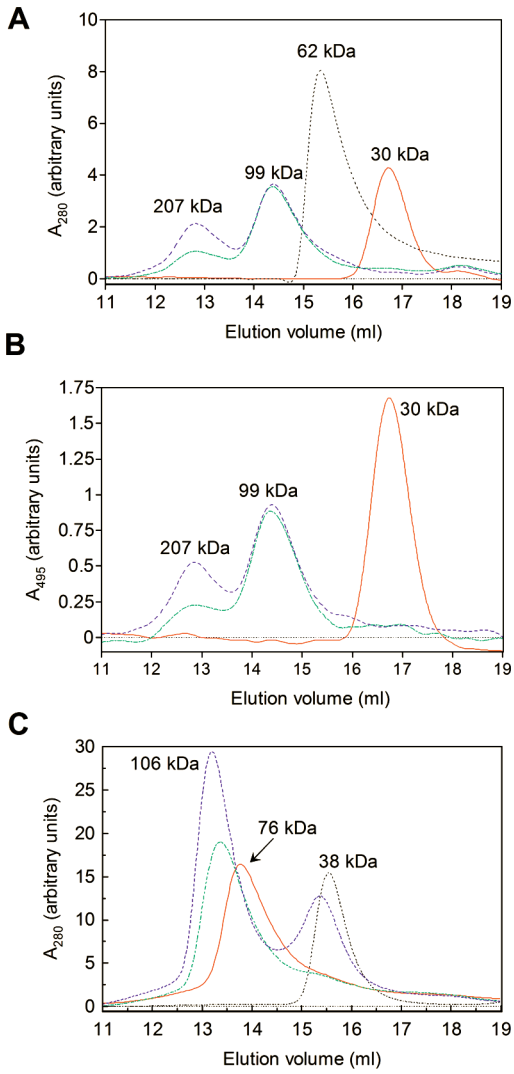

FIGURE 3: Gel-filtration analysis of full-length *Eco*31I and its N-terminal domain, *Eco*31I-N. Elution profiles of *Eco*31I and the 19/21-DNA-Fluo were observed either at 280 nm (A, DNA and protein) or at 495 nm wavelengths (B, DNA only). Black short dash line, elution of free *Eco*31I (5.0 μM); red line, elution of free DNA (0.5 μM); green dash-dot line, elution of *Eco*31I−DNA (1.0−0.5 μM) complex; blue dash line, elution of *Eco*31I−DNA (2.0−0.5 μM) complex. C, Gel-filtration of *Eco*31I-N and its complexes with specific 37/43-DNA. Black line, elution of free *Eco*31I-N (5.0 μM); red line, elution of free DNA (1.0 μM); green dash-dot line, elution of (*Eco*31I-N)−DNA (1.0−1.0 μM) complex; blue dash line, elution of (*Eco*31I-N)−DNA (4.0−1.0 μM) complex. The numbers above the peaks denote the apparent molecular mass values, calculated by interpolating measured elution volumes onto the calibration curve.

corresponding to the molecular masses of 99 kDa and 207 kDa (Figure 3). The 99 kDa species matches the 1 + 1 *Eco*31I−DNA complex (62 kDa + 30 kDa = 92 kDa). The 207 kDa peak is characterized by the same enzyme to DNA proportion (1:1) as the 99 kDa species, as the absorbance ratio A_{280} (protein + DNA)/A_{495} (DNA only) is identical for both the 92 kDa and the 207 kDa peaks (Figure 3A,B). Therefore, the 207 kDa species most likely is a 2 + 2 complex (92 kDa × 2 = 184 kDa). Of note is that,even at ≥0.5 μM concentrations of enzyme and DNA, only part of DNA and enzyme participate in this 207 kDa complex.

When *Eco*31I-N was applied on the gel-filtration column, its apparent molecular mass was found to be 38 kDa. This value is close to 29 kDa, the calculated mass of *Eco*31I-N monomer. To improve resolution of free *Eco*31I-N and its complexes with DNA on the gel-filtration column, we employed a longer specific duplex, specific 37/43-DNA. Free specific 37/43-DNA eluted at a volume corresponding to 76 kDa, a value that is 3-fold higher than the calculated molecular mass of 26 kDa. When the mixtures of the specific 37/43-DNA and *Eco*31I-N were applied on the column, a single peak corresponding to a molecular mass of 106 kDa was observed. This species corresponds to the 1 + 1 *Eco*31I-N complex with DNA (38 kDa + 76 kDa = 114 kDa) (Figure 3C).

Cleavage of Double-Stranded DNA by Eco31I. Type IIS REases cleave double-stranded DNA by several different mechanisms. The archetypal type IIS REase, *Fok*I, is a monomer in solution bearing a single catalytic center in the CTD. In order to cleave dsDNA, *Fok*I forms a transient dimer with two catalytic domains. The *Fok*I dimer is stabilized by simultaneous interaction with two recognition sites located on the same DNA molecule *in cis* (*29*), thus *Fok*I cleaves two-site plasmid substrates with much higher rates than substrates bearing only a single recognition site. The model of *Fok*I dimerization is also supported by structural data (*42*) and sigmoidal dependence of DNA cleavage rate on enzyme concentration observed under steady-state reaction conditions (*29, 43*). Mechanism of transient dimerization is also proposed for several other type IIS enzymes (*44*). However, some type IIS REases do not change their oligomeric state during DNA cleavage. One of such REases is *Bfi*I (*5*). Contrary to *Fok*I, *Bfi*I is a homodimer in solution, but it has only one active site at the dimerization interface (*26, 28*). It was demonstrated that *Bfi*I remains a dimer as it makes a double-strand break in DNA: the single active site of *Bfi*I acts sequentially, first on the bottom and then on the top DNA strands (*5*). Another characterized type IIS enzyme, *Mva*1269I, is a monomer in solution like *Fok*I, but it carries two active sites in a single polypeptide chain that introduces a double-strand break in the DNA duplex (*25*). In contrast, the type IIS REase *Bsp*MI is a stable homotetramer with four active sites that simultaneously binds two copies of cognate DNA and cleaves four phosphodiester bonds in a concerted reaction generating two double-strand breaks (*27*).

We have demonstrated that *Eco*31I, like *Fok*I, is a monomer in solution with a single active site located in the CTD (*8*). Thus, in order to cleave double-stranded DNA, *Eco*31I must either form a dimer on the DNA substrate or employ the same active site for consecutive cleavage of both DNA strands like *Bfi*I. To discriminate these possibilities, we performed kinetic analysis of DNA cleavage by *Eco*31I under multiple-turnover and transient reaction conditions.

First, we tested if *Eco*31I, like most characterized type IIS REases, requires simultaneous interaction with two copies of its recognition sequence for effective DNA cleavage. It was shown previously that all REases that bind two copies of recognition site prior to DNA hydrolysis cleave the supercoiled two-site plasmid substrates much more rapidly (up to 400-fold) than the single-site plasmids (*11, 29, 45–48*). Thus, we determined *Eco*31I DNA hydrolysis rates on supercoiled plasmid substrates with one and two copies of the recognition site (plasmids pUC19 and pEco31I-2 respectively, see Experimental Procedures for details).

The first set of experiments was performed under multiple-turnover reaction conditions (10 nM plasmid substrate, 0.4

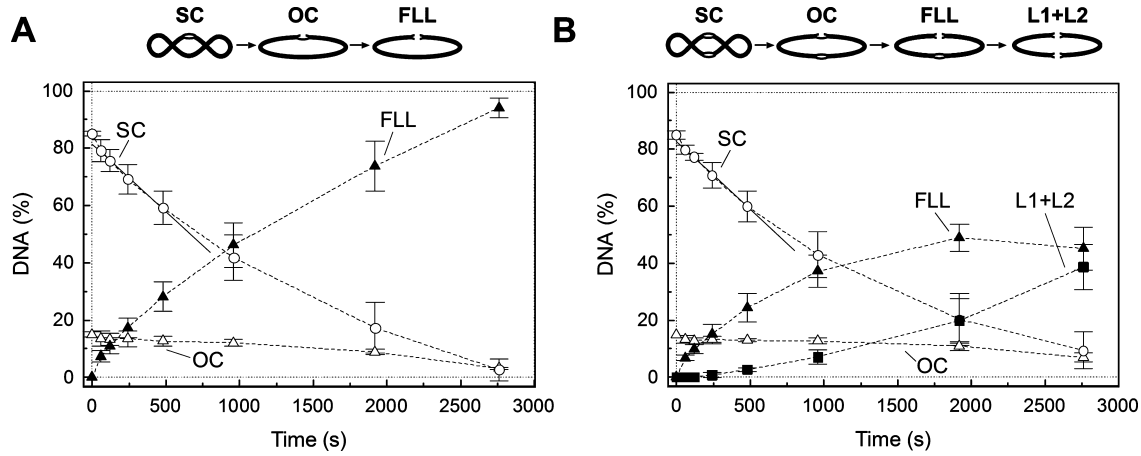

FIGURE 4: Multiple-turnover *Eco*31I reactions on plasmids with one and two recognition sites. The reactions contained 10 nM plasmid DNA and 0.4 nM of *Eco*31I monomer. The plasmids were pUC19 (one *Eco*31I site) for A, and pEco31I-2 (two *Eco*31I sites) for B. Samples were quenched with EDTA + SDS and analyzed as described in Experimental Procedures to determine the amounts of the following forms of the DNA: supercoiled DNA (SC), open circles; open-circular DNA (OC), open triangles; linear DNA cut at one *Eco*31I site (FLL), filled triangles; and, only in B, linear DNA cut at both *Eco*31I sites (L1 + L2), filled squares. Cartoons above the graphs schematically depict various forms of plasmid DNA that are formed during *Eco*31I reactions. Solid lines are linear fits to the initial phase of the SC plasmid cleavage profile that gave $v_{0(1\text{-site})} = 0.0046 \pm 0.0005$ nM/s^{-1} and $v_{0(2\text{-site})} = 0.0046 \pm 0.0005$ nM/s^{-1}.

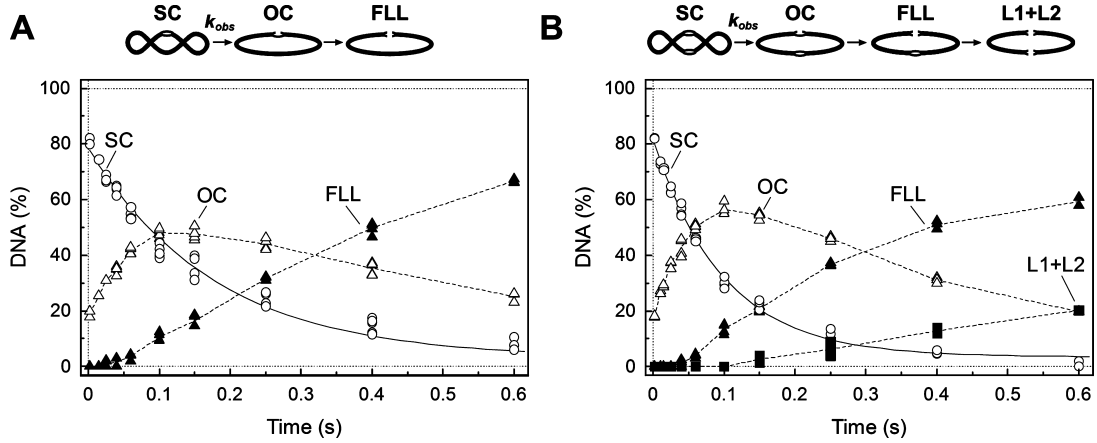

FIGURE 5: Single-turnover *Eco*31I reactions on plasmids with one and two recognition sites. The reactions were performed by mixing solution of Mg-acetate (final concentration 10 mM) with the preincubated mixture of enzyme and plasmid DNA (final concentrations 20−40 nM *Eco*31I monomer and 5 nM DNA) in a quench-flow device. The plasmids were pUC19 (one *Eco*31I site) for A, and pEco31I-2 (two *Eco*31I sites) for B. Samples were quenched with 6 M guanidinium chloride and analyzed as described in Experimental Procedures to determine the amounts of the following forms of the DNA: supercoiled DNA (SC), open circles; open-circular DNA (OC), open triangles; linear DNA cut at one *Eco*31I site (FLL), filled triangles; and, only in B, linear DNA cut at both *Eco*31I sites (L1 + L2), filled squares. Cartoons above the graphs schematically depict various forms of plasmid DNA that are formed during *Eco*31I cleavage of the one- and two-site plasmids. Solid lines are single-exponential fits to the supercoiled substrate cleavage data that gave $k_{obs(1\text{-site})} = 5.6 \pm 0.5$ s^{-1} and $k_{obs(2\text{-site})} = 10.1 \pm 0.7$ s^{-1}.

nM *Eco*31I, enzyme/DNA ratio 1:25). *Eco*31I cleaved both plasmid substrates with equal initial rates ($v_{0(1\text{-site})} \approx v_{0(2\text{-site})} = 0.0046$ nM/s, Figure 4A,B). In both cases the intact supercoiled substrate (SC) was directly converted into the full-length linear product with a single double-strand break (FLL) without accumulation of any nicked reaction intermediate (OC) in the reaction mixture. This indicates that *Eco*31I cleaves both strands of the DNA substrate during the lifetime of the *Eco*31I−DNA complex. The linearized two-site plasmid (form FLL) was further converted into the final reaction products: two linear DNA fragments (L1 + L2). The maximum yield of the FLL reaction intermediate in the pEco31I-2 cleavage reaction (Figure 4B) was ~50%, indicating that *Eco*31I cleaves both sites on the pEco31I-2 substrate with similar rates. Next, we performed DNA cleavage experiments under transient reaction conditions with excess of enzyme over the substrate (Figure 5). Unlike the steady-state reactions, the transient DNA cleavage experiments reveal the resultant rate of enzyme−substrate association and the chemical step of substrate cleavage, i.e. the rapid reaction stages that precede dissociation of the enzyme−product complex. *Eco*31I cleaves the SC form of the two-site plasmid only 2-fold faster than the one-site plasmid ($k_{obs(2\text{-site})} = 10.1$ s^{-1}, $k_{obs(1\text{-site})} = 5.6$ s^{-1}, Figure 5), indicating that simultaneous interaction with two recognition site is not a prerequisite for DNA hydrolysis by *Eco*31I.

It was demonstrated that *Fok*I, an enzyme that requires dimerization for DNA cleavage, displays a nonlinear dependence of the steady-state reaction rate on enzyme concentration: the reaction rates increase more steeply than expected for a linear dependence (*29, 43*). In contrast, *Bfi*I, which does not change its oligomeric state during the DNA cleavage reaction, displays an ordinary linear dependence of the steady-state reaction rate on enzyme concentration (*5*). To test if *Eco*31I forms a transient dimer during DNA hydrolysis, we performed *Eco*31I cleavage of one- and two-site

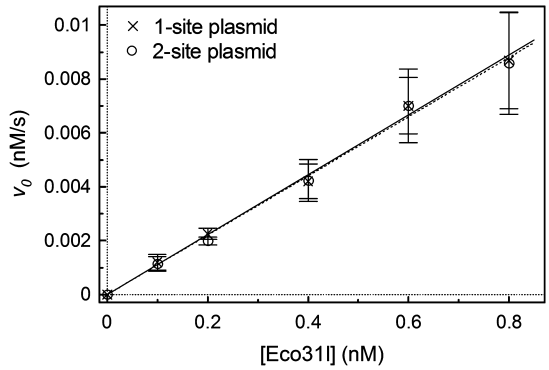

FIGURE 6: Dependence of the initial reaction rate on the *Eco*31I concentration. The reactions contained 10 nM plasmid DNA in the pH 7.9 reaction buffer at 25 °C and the indicated concentration of *Eco*31I. The plasmid DNA contained either one *Eco*31I recognition site (pUC19, crosses) or two sites (pEco31I-2, open circles). For each reaction, the initial rate for substrate utilization was measured as in Figure 4, and the values were plotted against the enzyme concentration. The lines illustrate the linear increase in reaction rate with enzyme concentration according to equation $v_0 = A \times$ [*Eco*31I], where slope coefficient A for both plasmids equals 0.011 $\pm$ 0.001 s^{-1}.

plasmid substrates across a range of enzyme concentrations, keeping the substrate concentration in the 10- to 100-fold excess over the enzyme. The determined multiple-turnover DNA cleavage rates for both substrates are summarized in Figure 6. It is evident that reaction rates for both substrates linearly depend on the enzyme concentration, indicating that either *Eco*31I does not change its oligomeric state upon DNA binding and cleavage or *Eco*31I forms a stable DNA-bound oligomer already at 0.1 nM, the lowest protein concentration used in the multiple-turnover experiments (Figure 6).

DISCUSSION

Domain Organization of Eco31I. We employed limited proteolysis with three different proteases to map the domain organization of type IIS REase *Eco*31I, which belongs to the "short-distance cutters". The initial *Eco*31I cleavage pattern with all proteases was represented by two predominant bands (Figure 1). Combined molecular masses of the fragments corresponded to the molecular mass of the full-length *Eco*31I. These observations imply the two-domain organization for *Eco*31I, as well as the presence of an interdomain linker easily accessible to proteases. N-terminal sequencing of the major protein fragments revealed that the smaller proteolytic fragment in all cases corresponds to the N-terminal part of *Eco*31I and the larger fragment to the C-terminal part. We also performed digestion of enzyme−DNA complexes, as cognate DNA may provide steric protection for the protein regions involved in DNA binding (*34*). Our finding that cognate DNA impeded proteolytic degradation of the initially released NTD of *Eco*31I (Figure 1C,D) strongly suggests that NTD is responsible for cognate DNA recognition. The cognate DNA binding function of the NTD was further confirmed by gel-mobility shift assay (Figure 2C,D), where NTD displayed strong specific interaction with a DNA duplex containing the *Eco*31I recognition site. Moreover, gel-filtration studies confirmed that the NTD of *Eco*31I binds DNA as a monomer (Figure 3C).

Noteworthy, apparent concentrations at which 50% of DNA was bound for the full-length *Eco*31I and the *Eco*31I-N are approximately the same, 5 nM (Figures 2A, 2C), indicating that the C-terminal domain of *Eco*31I does not contribute to cognate DNA binding by this enzyme. On the other hand, the CTD and possibly the interdomain region of the full-length *Eco*31I contribute to formation of nonspecific protein−DNA complexes, as the nonspecific complexes were not observed with the *Eco*31I-N (Figure 2D).

Despite identification of a HNH nuclease-like active center in the C-terminal part of *Eco*31I (*8*), we were not able to demonstrate any catalytic activity of the isolated C-terminal proteolytic fragment of *Eco*31I. Most likely, the CTD of *Eco*31I requires interactions with other parts of the full-length enzyme in order to catalyze DNA hydrolysis. This contrasts with the properties of isolated catalytic domains of other type IIS REases, including *Fok*I (*35*), *Mnl*I (*9*), *Bfi*I (*36*) and *Bpu*JI (*49*) that all displayed detectable nonspecific nucleolytic activity.

Oligomeric State of Eco31I. Dimerization of *Eco*31I on DNA was suggested based on gel-filtration results (*8*) and Figure 3A,B. The *Eco*31I−DNA complexes elute in two peaks, one corresponding to the enzyme monomer bound to DNA (the 1 + 1 complex), and the heavier species that presumably corresponds to a protein dimer interacting with two DNA duplexes (the 2 + 2 complex). However, at all enzyme−DNA ratios tested, only a fraction of enzyme was present in the 2 + 2 complex, though both DNA and enzyme were used at high concentrations (500−1000 nM, ref *8* and Figure 3A,B). This raises a question if the 2 + 2 complex is the catalytically active form of *Eco*31I in the DNA cleavage reactions performed with 0.1−1.0 nM enzyme (Figure 4 and Figure 6).

Kinetic Studies of Eco31I Reactions. A single HNH nuclease-like active site was identified in the C-terminal part of *Eco*31I using bioinformatic methods and mutational analysis (*8*). Gel-filtration studies indicate that *Eco*31I is a monomer in solution at concentrations as high as 5 μM (Figure 3A and ref *8*). A question that then arises is how *Eco*31I cleaves two DNA strands downstream of its recognition site. One possibility is that *Eco*31I dimerizes on cognate DNA, forming a complex with two catalytic centers capable of double-stranded DNA cleavage. This *Eco*31I dimer could interact with two copies of the recognition sequence via the two DNA binding domains, as demonstrated for the type IIS enzyme *Fok*I (*50*). Alternatively, a catalytic dimer of *Eco*31I could be formed on a single copy of the recognition sequence with only one of the two NTDs involved in DNA binding. The third possibility is that *Eco*31I interacts with DNA as a monomer via the NTD and sequentially cleaves the first and the second DNA strands, with both reactions occurring in the same active site of the enzyme, as suggested for REase *Bfi*I (*5*) and homing endonuclease I-*Tev*I (*51*).

Kinetic analysis of *Eco*31I reactions argues against the reaction mechanism akin to *Fok*I, i.e. dimerization of two DNA-bound *Eco*31I monomers. Indeed, *Eco*31I cleaves one- and two-site DNA substrates with similar rates, both under multiple-turnover conditions (v_0 values determined for one- and two- site substrates are identical, Figures 4 and 6) and in the single-turnover reactions (k_{obs} for the one-site and two-site plasmids equal 5.6 s^{-1} and 10.1 s^{-1}, Figure 5). Of note is that 2-fold acceleration of the two-site plasmid cleavage compared to the one-site plasmid observed in the transient kinetic experiments could be due to the fact that hydrolysis

of the two-site plasmid is simultaneously performed by two enzyme molecules bound to each of the two recognition sites, while cleavage of the one-site plasmid is performed by a single enzyme molecule bound to the solitary recognition site, as previously demonstrated for the dimeric mutant of endonuclease *Bse*634I (*46*). Thus, under both multiple-turnover and transient reaction conditions *Eco*31I cleaves the one- and two-site DNA substrates with equal rates. This indicates that *Eco*31I acts on individual copies of the recognition sequence and does not require formation of synaptic complexes with two cognate DNA sites.

The ability to act on lone copies of the DNA recognition site distinguishes *Eco*31I from most type IIS REases studied to date, including *Fok*I, *Bsg*I, *Bsp*MI and others (*29*). On the other hand, *Eco*31I is akin to the previously characterized type IIS restriction enzymes cutting at a short distance from the recognition site (≤5 bp), including *Msp*1269I, *Bsm*BI, *Sap*I and *Bsa*I (*25, 29*). Kinetic studies of all these REases suggested that none of these enzymes require interactions with two copies of the recognition sequence for DNA cleavage.

Furthermore, *Eco*31I displays a linear dependence of the multiple-turnover reaction rate on enzyme concentration for both one- and two-site plasmid substrates (Figure 6). This result is also inconsistent with *Eco*31I dimerization during the DNA cleavage reaction. Indeed, if *Eco*31I subunit bound to the recognition site recruits a second monomer from free solution, DNA cleavage under multiple-turnover reaction conditions ([E] ≪ [S]) would be possible only if the free *Eco*31I monomer has a higher affinity for the DNA-bound monomer than for an unoccupied recognition site. Otherwise, under [E] ≪ [S] conditions *Eco*31I dimers would be formed only on a minute fraction of DNA sites, as the great majority of the enzyme would interact with DNA in the monomeric form. In addition, the multiple-turnover reaction rates with both substrates would display a nonlinear dependence on enzyme concentration. Thus, equal rates of one- and two-site plasmid cleavage and linear variation of the reaction rate observed in our experiments (Figure 6) imply that *Eco*31I presumably does not change its oligomeric structure upon DNA binding and cleaves double-stranded DNA as a monomer. An unlikely alternative is that *Eco*31I forms stable DNA-bound dimers already at 0.1 nM, the lowest enzyme concentration used in our multiple-turnover experiments (Figure 6).

Kinetic analysis of *Eco*31I is in apparent conflict with the gel-filtration experiments that suggested dimerization of *Eco*31I on two copies of cognate DNA (Figure 3). A possible explanation for this discrepancy is that the 2 + 2 *Eco*31I−DNA complex identified by gel-filtration at micromollar enzyme and DNA concentrations is not a catalytically competent species of *Eco*31I, but rather a nonspecific *Eco*31I−DNA complex formed due to nonspecific protein−DNA or protein−protein interactions. Indeed, nonspecific *Eco*31I−DNA complexes were detected by gel-shift experiments already at 10−25 nM concentrations of *Eco*31I (Figure 2A,B).

Mechanism of Double-Stranded DNA Cleavage. Based on the kinetic studies, we conclude that *Eco*31I most likely remains a monomer as it binds DNA and cleaves two phosphodiester bonds downstream of its recognition site. The ability to perform site-specific cleavage of double-stranded DNA employing a single recognition site was previously proposed only for the type IIS enzyme *Bfi*I and the homing endonucleases I-*Tev*I and I-*Bmo*I (*5, 51, 52*).

An obvious problem for all these nucleases is that scissile phosphodiester bonds located in the two DNA strands are separated by a significant distance, thus after hydrolysis of the first phosphodiester bond, the enzyme−DNA complex must undergo a dramatic conformational rearrangement required to transfer the catalytic center to the second phosphodiester bond. Moreover, hydrolysis of phosphodiester bonds in the opposite DNA strands might seem to require a 180° rotation of the catalytic center between the two reactions, to match the antiparallel polarity of the second DNA strand. This rotation is unnecessary only for *Bfi*I, as its single active site is located on the 2-fold symmetry axis between the protein subunits (*5, 28*).

Kinetic studies indicated that all these enzymes cut the two DNA strands in a strictly defined order, being able to cleave the top DNA strand only after hydrolysis of the bottom one (*5, 51, 52*). Studies of I-*Tev*I and I-*Bmo*I also showed that after cleavage of the first DNA strand these proteins induce a bend of the DNA substrate near the cleavage site that promotes cleavage of the second DNA strand (*51, 52*). However, a strictly defined order for cleavage of two DNA strands is unlikely for the REase *Eco*31I. Previous studies of *Eco*31I revealed that, depending on the sequences surrounding the recognition site, wild-type *Eco*31I and certain active site mutants preferentially cleave either the top or the bottom DNA strand (*8*).

Inspection of the reaction profile of the single-turnover *Eco*31I reaction on the one-site plasmid (Figure 5A) reveals that *Eco*31I hydrolyzes the first and the second DNA strands with comparable rates, as the amount of the nicked reaction intermediate does not exceed 50% of all products. This suggests that rearrangement of the *Eco*31I−DNA complex occurring upon cleavage of the first DNA strand does not limit the rate of the second DNA strand cleavage. In that respect *Eco*31I differs from the single-site REase *Bfi*I that cleaves the bottom DNA strand much more rapidly than the top one (*5*).

Conclusions. For the first time we report the domain organization of a REase belonging to the group of "short distance cutters". We show here that the type IIS enzyme *Eco*31I consists of the N-terminal domain responsible for DNA recognition and the C-terminal domain that contains the catalytic center related to HNH nucleases. Though gel-filtration studies indicate that *Eco*31I is able to form a homodimer on cognate DNA, kinetic analysis of *Eco*31I reactions argues against the functional significance of *Eco*31I dimerization and instead favors the model where *Eco*31I remains a monomer as it binds and cleaves the double-stranded DNA substrate. The domain organization and reaction mechanism established for *Eco*31I should be common for a group of evolutionary related REases *Alw*26I, *Bsa*I, *Bsm*AI, *Bsm*BI and *Esp*3I that recognize DNA sequences bearing the 5′-GTCTC pentanucleotide (*8, 31*).

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