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Resolution of the EcoRII restriction endonuclease-DNA complex structure in solution using fluorescence spectroscopy

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ABSTRACT

The X-ray structure for the type IIE EcoRII restriction endonuclease has been resolved [X.E. Zhou, Y. Wang, M. Reuter, M. Mucke, D.H. Kruger, E.J. Meehan and L. Chen. Crystal structure of type IIE restriction endonuclease EcoRII reveals an autoinhibition mechanism by a novel effector-binding fold. J. Mol. Biol. 335 (2004) 307–319.], but the structure of the R.EcoRII–DNA complex is still unknown. The aim of this article was to examine the structure of the pre-reactive R.EcoRII–DNA complex in solution by fluorescence spectroscopy. The structure for the R.EcoRII–DNA complex was resolved by determining the fluorescence resonance energy transfer (FRET) between two fluorescent dyes, covalently attached near the EcoRII recognition sites, that were located at opposite ends of a lengthy two-site DNA molecule. Analysis of the FRET data from the two-site DNA revealed a likely model for the arrangement of the two EcoRII recognition sites relative to each other in the R.EcoRII–DNA complex in the presence of Ca^{2+} ions. According to this model, the R.EcoRII binds the two-site DNA and forms a DNA loop in which the EcoRII recognition sites are 20±10 Å distant to each other and situated at an angle of 70±10°.

1. Introduction

Type IIE restriction endonucleases (REases) are attractive models for studying the specific protein–DNA interactions accompanying complex genetic processes in which enzymes simultaneously interact with DNA at several sites. Type IIE REases exhibit the particular property that they have to simultaneously bind two copies of their palindromic DNA recognition sequence for efficient DNA cleavage, where one copy is the target for cleavage and the other serves as an allosteric effector [1].

The EcoRII restriction endonuclease (R.EcoRII) was the first documented example of a Type IIE enzyme [2]. It is a homodimeric protein. R.EcoRII cleaves the ↓CCA/TGG sequence in DNA at both strands at the positions defined by the arrow. The low cleavage efficiency of R.EcoRII on DNA substrates with only a single or a few DNA recognition sites separated by >1000 bp was overcome by adding short duplexes with a single EcoRII recognition site (due to *trans*-interactions) [3]. A study of the cleavage by R.EcoRII of two distinct recognition sites on the same DNA molecule shows that cooperativeness between the EcoRII sites is achieved by bending or looping of the intervening DNA stretch (*cis*-interactions) [4]. Transmission electron microscopy provided direct evidence that R.EcoRII mediates a loop formation [5]. Further studies revealed that the enzyme-substrate active complex includes two subunits of R.EcoRII interacting with two DNA recognition sequences [4,6]. Recently, according to a kinetic study of the R.EcoRII cleavage of plasmids containing a single, two or three recognition sites, it was suggested that R.EcoRII requires simultaneous binding of three rather than two recognition sites *in cis* to achieve concerted DNA cleavage at a single-site [7]. Atomic force microscopy provided direct visualization and characterization of the synaptic protein–DNA complexes involving a two-loop structure with three DNA binding sites [8]. The X-ray structure of R.EcoRII was determined [9]. REase EcoRII contains an N-terminal effector-binding domain and a C-terminal endonuclease-like domain [9,10]. The putative catalytic site of R.EcoRII is located in the C-terminal domain and is spatially blocked by the N-terminal domain. The removal of the N-terminal effector-binding domain of R.EcoRII converts this type IIE enzyme into a very active Type IIP enzyme [10]. DNA binding studies indicate that the isolated C-terminal domain exists as a dimer that binds a single cognate DNA molecule in the presence of the analog cofactor, Ca^{2+} ions, whereas the N-terminal domain exists as a monomer that also binds a single copy of cognate DNA, but in a Ca^{2+}-independent manner [11]. It has been suggested that the full-length R.EcoRII contains three putative DNA binding interfaces: one at the C-terminal domain dimer and two at each of the N-terminal domains [11,12]. The distance between the EcoRII recognition sites and their arrangement relative to

Abbreviations: REase, restriction endonuclease; EMSA, electrophoretic mobility shift assay; ON, oligodeoxynucleotide; **FAM**, 5(6)-carboxyfluorescein; **TAMRA**, 5(6)-carboxytetramethylrhodamine; FRET, fluorescence resonance energy transfer; C_{DNA}, DNA concentration.

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each other in this complex are not known. The structural organization of the pre-reactive R.EcoRII–DNA complex, which is formed in the presence of a cofactor, Mg^{2+} ions, is of a special interest. It was shown that substitution of Ca^{2+} ions for Mg^{2+} ions results in inhibition of DNA cleavage by R.EcoRII, but preserves the ability of R.EcoRII to form the pre-reactive R.EcoRII–DNA complex [13].

The use of fluorescence resonance energy transfer (FRET) can provide information on the distance between a donor and an acceptor dye in the range of 10 to 100 Å. To study the structure of the DNA-protein complexes, 5(6)-carboxyfluorescein, **FAM**, (as the donor) and 5 (6)-carboxytetramethylrhodamine, **TAMRA**, (as the acceptor) were used [14]. Using FRET allowed us to determine the distance between the DNA ends and, as a result, to distinguish the two U-shaped DNA global structures induced by the integration of the host factor upon binding with intact and nicked DNA [15]. The study of energy transfer between **FAM** and **TAMRA**, which were attached near the two operator DNA sites, about 130 bp apart, confirmed and extended the data about the geometry of the loop formed by the binding of the two operators by the LacI repressor [16]. FRET experiments allowed for the investigation of the DNA looping by the NgoMIV restriction endonuclease and the determination of the distance between the ends of the DNA molecule when bound to NgoMIV [17].

To determine the arrangement of the EcoRII recognition sites relative to each other within the R.EcoRII–DNA complex, the data from a FRET analysis of the R.EcoRII–DNA complex were studied using two fluorescent dyes that were covalently attached near the EcoRII recognition sites, which were located at opposite ends of the lengthy two-site DNA molecule. The R.EcoRII–DNA complex was formed in the presence of the Ca^{2+} ions to promote the formation of a pre-reactive R.EcoRII–DNA complex, which is hypothesized to resemble the same REase-DNA complex in the presence of cofactor Mg^{2+} ions prior to a reaction. A suggested model describing the arrangement of the two EcoRII recognition sites relative to each other in the complex of R.EcoRII with a two-site DNA is shown.

2. Experimental

2.1. Chemicals and enzymes

(γ-^{32}P)-ATP (1000 Ci/mmol) was from Izotop (Russia). R.EcoRII was overexpressed as an N-terminally His_6-tagged protein and purified by chromatography on a nickel chelate column as previously described [18]. The R.EcoRII dimer concentration was determined by Bradford assay (2.3 μM). T4 polynucleotide kinase was from MBI Fermentas (Lithuania). The buffers A–D, prepared with Milli-Q water, were compositions of the following solutions: A — 40 mM Tris–HCl at pH 7.6 and 50 mM NaCl; B — buffer A containing 5 mM $MgCl_2$ and 7 mM DTT; C — buffer A containing 5 mM $CaCl_2$ and 7 mM DTT; D — buffer A containing 7 mM DTT and 0.2 mM EDTA and E — 60 mM Tris–HCl at pH 8.5, 25 mM KCl, 10 mM 2-mercaptoethanol and 0.1% Triton X-100.

Oligonucleotides (ONs) were from Syntol (Russia) (Table 1). The fluorescent labels **FAM** and **TAMRA** were introduced to the 5′-end or 3′-end of the ONs via an aminoalkyl linker containing six methylene groups. ^{32}P-5′-phosphorylation of the ONs was carried out using T4-polynucleotide kinase and [γ-^{32}P]-ATP. ON concentrations were determined spectrophotometrically. Extinction coefficients (ε_{260}) of unmodified ONs at 260 nm were calculated according to a published protocol [19]. For the modified ONs, ε_{260} were calculated as the sum of ε_{260} of unmodified ON and ε_{260} for 5′-FAM (20,960 M^{-1} cm^{-1}), 3′-FAM (21,000 M^{-1} cm^{-1}) or 5′-TAMRA (29,100 M^{-1} cm^{-1}) (www.idtdna.com).

2.2. Preparation of fluorescence-labeled DNA segments

The 327–339 bp linear DNAs (Table 2) were obtained by PCR amplification. The plasmid pCAL7/nH was used as a DNA template. The 5′-**TAMRA**-$(CH_2)_6$-CAT CTA CCT G**CC TGG** ACA G, 5′-**TAMRA**-$(CH_2)_6$-ACC TG**C CTG G**AC AGC ATG G, 5′-ACC TG**C CTG G**AC AGC ATG G and 5′-**TAMRA**-$(CH_2)_6$-CTG **CCT GG**A CAG CAT GGC were used as forward primers. The 5′-**FAM**-$(CH_2)_6$-ACG TGG CTG G**CC TGG** TTC, 5′-**FAM**-$(CH_2)_6$-GCT GG**C CTG G**TT CAC CAC and 5′-GCT GG**C CTG G**TT CAC CAC were used as reverse primers. PCR amplification was carried out in 80 μl of buffer E containing 10 U of DNA-polymerase Taq, 1.4 μg of DNA template, 150 pmol of primers and a 2 mM mixture of the four dNTPs (0.5 mM of each). (α-^{32}P)-ATP was added to introduce a ^{32}P-label onto the DNAs. All PCR products migrated as individual bands in 0.5% agarose gels. PCR products were purified using a "Promega" kit (#A7170, Wizard PCR Preps DNA purification system).

2.3. DNA cleavage by R.EcoRII

Cleavage of **14^A/14^T** and **14^A/5′-FAM-14^T** duplexes (Table 1) was determined spectrophotometrically [20]. The experiment was repeated three times.

2.4. FRET measurements

Fluorescence measurements were performed with a "PTI technology" spectrofluorometer or with a spectrofluorometer "FluoroMax™" (SPEX^R) equipped with a thermostated cell holder in 150–3000 μl of buffer C. Aliquots of R.EcoRII ($C_{R.EcoRII}$ 0–500 nM) were added to DNA (C_{DNA} is

Table 1
Oligodeoxynucleotide sequences

14^A	5′-AGAG**CCAGG**TTGGC
14^T	5′-GCCAA**CCTGG**CTCT
5′-FAM-14^T	5′-**FAM**-GCCAA**CCTGG**CTCT
18^A	5′-TCAGAG**CCAGG**TTGGCTC
18^T	5′-AGTCTC**GGTCC**AACCGAG
5′-FAM-18^A	5′-**FAM**-TCAGAG**CCAGG**TTGGCTC
5′-TAMRA-18^A	5′-**TAMRA**-TCAGAG**CCAGG**TTGGCTC
5′-TAMRA-18^T	5′-**TAMRA**-GAGCCAA**CCTGG**CTCTGA

FAM – donor – 5(6)-carboxyfluorescein; **TAMRA** – acceptor – 5(6)-carboxytetramethylrhodamine.