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Interaction of EcoRII restriction and modification enzymes with synthetic DNA fragments. VI. The binding and cleavage of substrates containing nucleotide analogs

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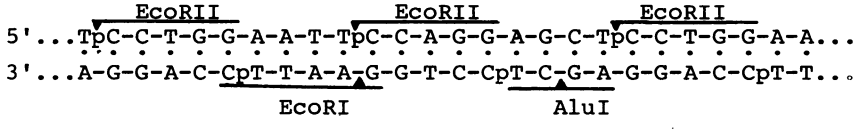
ABSTRACT

The present study deals with the binding and cleavage by EcoRII endonuclease of concatemer DNA duplexes containing EcoRII recognition sites (5'...↓CC$^{A}_{T}$GG) in which dT is replaced by dU or 5-bromodeoxyuridine, or 5'-terminal dC in the dT-containing strand is methylated at position 5. The enzyme molecule is found to interact with the methyl group of the dT residue of the DNA recognition site and to be at least in proximity to the H^5 atom of the 5'-terminal dC residue in dT-containing strand of this site. Modification of any of these positions exerts an equal effects on the cleavage of both DNA strands. Endonuclease EcoRII was found to bind the substrate specifically. At the same time modification of the bases in recognized sequence may result in the formation of unproductive, though stable, enzyme-substrate complexes.

INTRODUCTION

Selective modifications of DNA heterocyclic bases is a productive approach making it possible to ascertain the role of individual fragments of bases in protein-nucleic acid interactions [1-6]. We applied this approach in investigating EcoRII restriction endonuclease which recognizes in DNA the sequence. 5'...↓C-C*-A-G-G-... [2]. In this paper we examined the specific 3'...-G-G-T-C*-C↑...
binding and cleavage by endonuclease EcoRII of concatemer DNA duplexes containing the natural and modified recognition sites of this enzyme which recur every 9 base pairs:



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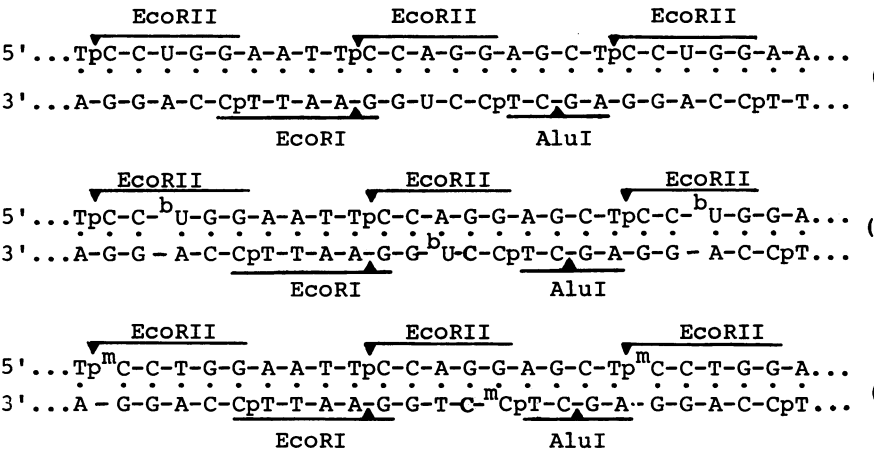

where ^{b}U and ^{m}C are 5-bromodeoxyuridine and 5-methyldeoxycytidine respectively. DNA duplexes I-IV contain also EcoRI and AluI recognition sites repeated every 18 base pairs. We modified EcoRII recognition sites by replacing dT in the central AT-pair with dU or $br^{5}dU$ (polymers II and III respectively) and by methylation of 5'-terminal dC in the dT-containing strand at position 5 (polymer IV). We should note here that methylase EcoRII introduces the methyl groups to positions 5 of two other dC residues neighbouring to the central AT-pair (see above, indicated by an asterisk) [2]. Polymer I is constructed [7,8] on the basis of the two nonadeoxynucleotides d(C-C-A-G-G-A-G-C-T) (9A) and d(C-C-T-G-G-A-A-T-T) (9T). Polymers II-IV are constructed from 9A and d(C-C-U-G-G-A-A-T-T) (9U), 9A and d(C-C-^{b}U-G--G-A-A-T-T) (9B), 9A and d(^{m}C-C-T-G-G-A-A-T-T) (9M), respectively. The specific feature of these substrates is that the nucleotide analogs in the EcoRII recognition sites are arranged in checkered pattern. A study of the interaction of endonuclease EcoRII with substrates II-IV enabled us to obtain information about the contacts of this enzyme with individual nucleotides in the recognized sequence. Besides, the use of substrates II-IV made it possible to study the effect of modificiations of one of the two strands of the recognition site on the cleavage of each of these strands. Interaction of the enzyme with DNA duplex V, which does not contain EcoRII recognition site,

5'...T-G-C-A-C-A-T-G-T-G-C-A-C-A-T-G-T-G-C-A-C...
3'...A-C-G-T-G-T-A-C-A-C-G-T-G-T-A-C-A-C-G-T-G... (V),

was also studied.

MATERIALS AND METHODS

Nonanucleotides. 9A and 9T [7,8] 9U, 9B and 9M [9] were synthesized as described earlier. The primary structure of all the nonanucleotides was confirmed by the method of Maxam and Gilbert [10]. To prove the nucleoside composition of 9B, 9U, 9M they were hydrolyzed by a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase [11]. The hydrolysis products were analysed by HPLC on Zorbax C8 4.6x250 mm columns ("Chrompack", The Netherlands) in a chromatographer "Tracor" (The Netherlands). 0.1 M ammonium acetate in 12% methanol was used as a mobile phase, the elution rate was 1.5 ml/min. The chromatograms were computed by "Hewlett-Packard 3390A" electronic integrator. The molar extinction coefficients at 260 nm were taken as 7350 (dC), 11750 (dG), 8751 (dT), 15004 (dA), 10108 (dU), 5250 (br^5dU) and 5600 (m^5dC).

Enzymes T4 polynucleotide kinase, T4 DNA ligase and restriction endonucleases EcoRI and AluI were from NIKTI BAV (Novosibirsk, USSR), EcoRII was isolated in an electrophoretically homogeneous state as described previously [12]. The protein concentration was assayed by the Lowry method [13].

Synthesis of the substrates 5'-phosphorylated nonanucleotides p9A, p9T, p9U, p9B and p9M were obtained from 9A, 9T, 9U, 9B and 9M, respectively, using T4 polynucleotide kinase and ATP ("Serva", FRG) as described previously [11]. A similar procedure was applied for introducting the ^{32}P-label into the 5'-termini of nonanucleotides [7]. Polymers I-IV were synthesized by T4 DNA ligase from p9A and p9T (polymer I), p9A and p9U (polymer II), p9A and p9B (polymer III), p9A and p9M (polymer IV). 700 pmol of nonanucleotides were incubated with 10-20 act. units of T4 DNA ligase in 20 µl of 50 mM tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 1 mM ATP at 8-10°C for 12-18 h. The same method was used for obtaining DNA duplex V from d(pT-G-C-A-C-A-T-G) [14]. Plasmid DNA pBR 322 was isolated from E.coli B834/pBR 322 and kindly donated by

A.A. Oganov (Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences).

Cleavage of polymers I-IV by endonuclease EcoRII. The reaction was performed in 10 µl of 40 mM tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM dithiothreitol, 4% (v/v) glycerol (buffer 1), containing 7-8 act. units of EcoRII at 37°C. The substrate concentration per nucleotide residue (C_N) was 12.8 µM. The EcoRII activity unit was defined as the minimum amount of enzyme able to digest completely 1 µg of pBR 322 plasmid in 10 µl of buffer 1 at 37°C in 1 h.

Cleavage of polymers I-IV by endonucleases EcoRI and AluI. The substrate (C_N 12.8 µM) was incubated at 37°C in 10 µl of buffer 1 with 1 act. unit of AluI for 2 h or in 10 µl of 80 mM tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM dithiothreitol, 4% (v/v) glycerol with 2 act. units of EcoRI for 1 h.

Analysis of cleavage products. The reactions were stopped by addition EDTA to a concentration 10 mM. The products of cleavage of ^{32}P-labeled polymers I-IV were electrophoresed in a 20% polyacrylamide gel containing 7 M urea. Then the gel was autoradiographed and the radioactivity of the gel slices that corresponded to the intact substrate and the reaction products - nonanucleotides and their oligomers (k-mers) was determined by Cherenkov counting. These data were used for computing the relative mass content of each of the k-mers (M_k) and the amount of cleaved bonds as described previously [15].

Assay of endonuclease EcoRII binding to polymers I-V. The polymers (C_N 125 nM) were incubated with endonuclease EcoRII (1.4-280 nM per dimer, this enzyme consists of two identical subunits with a molecular mass of 44000 [12]) at 37°C for 10 min in 50 µl of 40 mM tris-HCl buffer, pH 7.6, containing 50 mM NaCl, 7 mM β-mercaptoethanol (buffer 2) in the presence of 50 µg/ml bovine serum albumin. The incubation mixtures were filtered through a stack of filters prewashed with 0.5 ml of buffer 2: nitrocellulose HAWP 0.45 µm ("Millipore"), "Whatman 3MM" paper and DEAE-paper "DE-81". Then the filters were washed with 1 ml of heated to 37°C buffer 2 and their radioactivity was determined by Cherenkov counting. The fraction of the labeled DNA duplexes retained on the nitrocellulose filter (R) was

calculated as a ratio of the radioactivity on nitrocellulose to the overall radioactivity on the nitrocellulose and DEAE-paper.

Studying the dissociation kinetics of enzyme-substrate complexes. The complexes of polymers I-V (C_N 125 nM) with endonuclease EcoRII (8 nM for polymers I-IV, 28 nM for polymer V) were incubated for 10 min at 37°C in the buffer 2 in the presence of 50 μg/ml bovine serum albumin. Then calf thymus DNA ("Biolar", Olaine, USSR) was added to a concentration of 1.1 mM per nucleotide residue, and the mixture was incubated at 37°C. 50 μl aliquots were taken at definite time intervals and analyzed on nitrocellulose filters as described above. In control experiment polymers I-IV were incubated with endonuclease EcoRII at 37°C in the same conditions without calf thymus DNA. The complexes were sustained throughout the experiments.

RESULTS

1. Synthesis of the substrates. Polymer I was obtained as described [8]. Nonanucleotides 9U, 9B and 9M were synthesized earlier [9]. To prove their structure 9U, 9B and 9M were sequenced [9] by the method of Maxam and Gilbert [10] and their nucleoside compositions were determined. For this purpose the products of complete hydrolysis of 9U, 9B, 9M by a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase were analyzed by HPLC under specially selected conditions ensuring effective separation of nucleosides dC, dG, dT, dA, m^5dC and br^5dU (Fig. 1a). By way of example, analysis of the

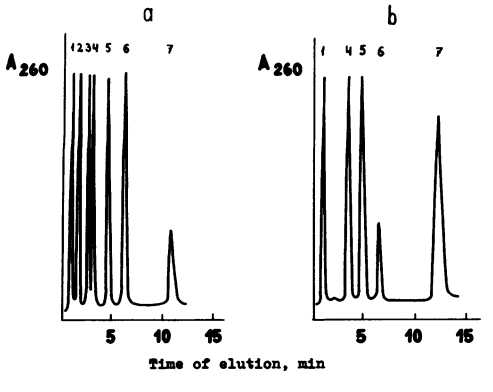

Fig. 1. HPLC of deoxynucleosides on a "Zorbax C8" column: a - the control mixture, b - the products of hydrolysis of nonanucleotide 9B by a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase. Peaks: 1 - dC, 2 - dU, 3 - m^5dC, 4 - dG, 5 - dT, 6 - br^5dU, 7 - dA.

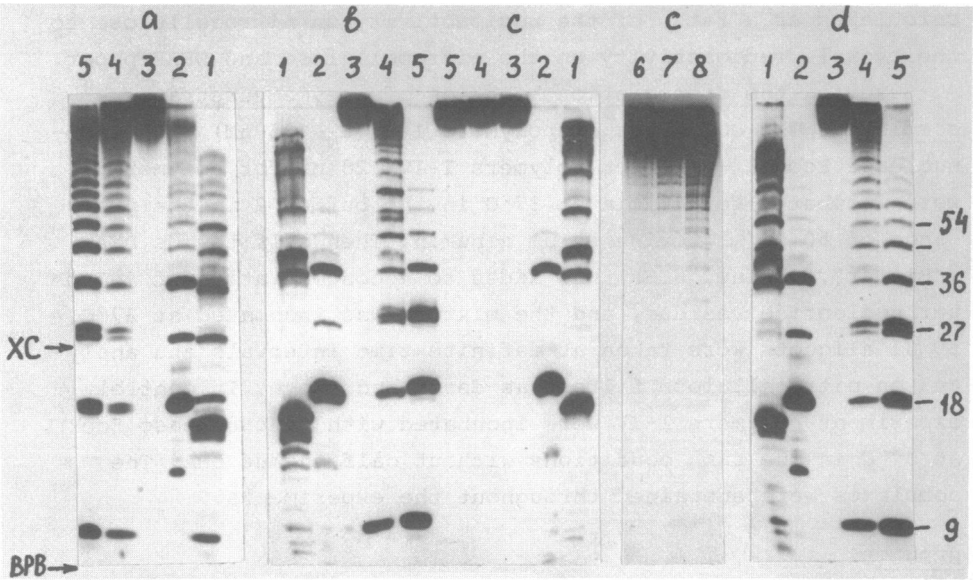

Fig. 2. Electrophoresis of the products of cleavage of polymers II (a), III (b), IV (c) and I (d) by endonucleases AluI (1), EcoRI (2) and EcoRII (4,5,6,7 and 8, that corresponds respectively to 2, 20, 30, 60 and 120 minutes of reaction). 3 - the initial substrates. Figures on the right indicate the lengths of the products (the numbers of nucleotide residues). XC and BPB are the positions of xylene cyanol and bromophenol blue, respectively.

products of 9B enzymatic hydrolysis is given (Fig. 1b). The nucleoside composition of the nonanucleotides agree with the anticipated ones: for 9U dC:dU:dG:dA:dT = 1.96:0.84:2.00:2.26: 2.28; for 9B dC:br^{5}dU:dG:dA:dT = 1.92:1.00:1.84:1.91:2.04; for 9 M m^{5}dC:dC:dG:dA:dT = 1.08:0.92:2.00:2.22:2.80.

Polymers II-IV were obtained by enzymatic ligation of corresponding 5'-phosphorylated nonanucleotides by the procedures evolved by us for the polymer I [8]. Ligation of nonanucleotide blocks by T4 DNA ligase proceeds in all the cases as effectively as during polymer I synthesis. The presence of 5'-terminal m^{5}dC in p9M does not interfere with the operation of T4 DNA ligase. As seen from Fig. 2, the synthesized substrates are formed by extended (200-1000-membered) polynucleotides. Like polymer I, polymers II-IV are effectively splitted by EcoRI and AluI restriction endonucleases with the formation of 18-membe-

red oligonucleotides as the main products (Fig. 2), which proves the structure of the synthesized substrates.

2. Cleavage of polymers II-IV by endonuclease EcoRII. As seen in Fig. 2, polymers II and III, like polymer I [8,15], are cleaved by EcoRII enzyme and yield nonanucleotides and 18, 27, 36, etc.-membered products. Polymer IV is not cleaved detectably under the same conditions. However, prolonged incubation (2 h) results in the formation of very small amounts of the same products as in the case of polymers I-III (Fig. 2). The compositions of the cleavage products (a sets of M_k values, see "Materials and Methods") of polymers I-III were calculated. No any predominance of polynucleotides with the length multiple to 18 was observed among these products. This fact indicates that there is no preferential cleavage of unmodified or modified strand for polymers II and III. In the case of polymer I (see also our previous paper [15]) and polymer III nonanucleotides prevail even at a low degree of hydrolysis. For polymer II the nonanucleotide content is appreciably lower. Analysis of the sets of M_k values according to the method described by us for polymer I [15] shows that both polymer III and polymer I are cleaved by EcoRII enzyme processively. In this type of cleavage the bound molecule of the enzyme may slide along the DNA duplex and effect a multiple cleavage of the substrate at several recognition sites. It is under this type of cleavage that the reaction mixture is enriched by the products of complete cleavage [16], which are the nonanucleotides for our substrates. Processivity of cleavage is less pronounced in the case of hydrolysis of polymer II. It is possible that like EcoRI [16], endonuclease EcoRII is capable of processive cleavage of only those substrates in which, like in polymers I-IV, the recognition sites are in very close proximity to one another. When the plasmid pBR 322 (which contains six EcoRII sites separated by hundreds of base pairs [17]) is cleaved by EcoRII endonuclease, the products of complete cleavage do not prevail at a low degree of hydrolysis (data not shown). This points to a distributive type of cleavage [16], in which a transition of enzyme molecule to another recognition site occurs only via complete dissociation of the enzyme-substrate complex.

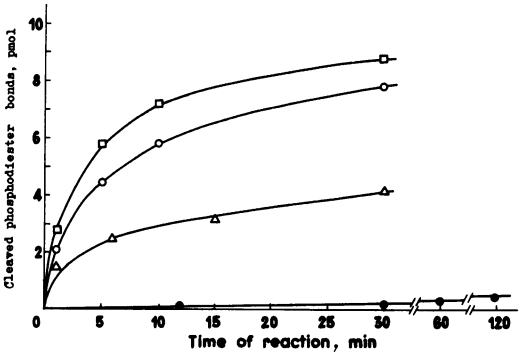

Fig. 3. Rates of cleavage of polymers I (o), II (△), III (□) and IV (●) by endonuclease EcoRII. Buffer I, C_N 12.8 μM, 7-8 act. units of EcoRII, 37°C.

The rates of EcoRII cleavage of substrates I-IV are compared in Fig. 3. Polymer II is cleaved more slowly than polymer I, whereas polymer III is cleaved slightly faster than polymer I. The rate of polymer IV cleavage is extremely low. The substitution of br^5dU for dT accelerates hydrolysis of the substrate also in the case of endonuclease MboI [3,4]. According to the data of Petruska and Horn [4], however, mammalian DNA in which br^5dU is substituted for all the dT residues is cleaved by EcoRII slightly more slowly than its non-modified analog. We may point to several possible reasons for the difference between these data [4] and those obtained by us. Firstly, in polymer III br^5dU is substituted for only those dT residues which are contained in the EcoRII recognition sites, and not for all of them. The substitution of br^5dU for all the dT residues in λ DNA reduces the rate of cleavage of this substrate by endonuclease SmaI, though the SmaI recognition site does not contain dT [6]. Secondly, polymers I and III are cleaved by EcoRII enzyme processively, whereas for the mammalian DNA used by the authors [3,4], like for plasmid pBR 322, distributive cleavage is to be expected. On our mind, different mechanisms of cleavage may result in different effects of base modifications on the rates of hydrolysis (see also Section 3). Thirdly, it should be noted that Petruska and Horn [4] did not identify the products of hydrolysis and did not determine the amount of cleaved bonds. The results of EcoRII cleavage of polymers II and IV are quite consistent with the data of Huang et al. [5] according to which this enzyme cleaves slowly the DNA of bacteriophage

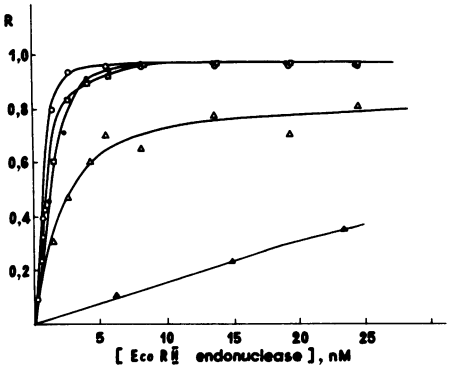

Fig. 4. Binding of endonuclease EcoRII to polymers I (o), II (△), III (□), IV (●) and V (▲) in buffer 2 at 37°C. C_N 125 nM.

PBSI where dU substitutes for all the dT residues, and does not cleave the DNA of bacteriophage XP12 where m^5dC substitutes for all the dC residues.

3. An investigation of EcoRII endonuclease binding to polymers I-V and dissociation kinetics of enzyme-substrate complexes

Endonuclease EcoRII complexation with the substrates I-IV, as well as with DNA duplex V lacking EcoRII recognition sites was studied on nitrocellulose filters in the absence of Mg^{2+}. Under these conditions the substrates I-IV are not cleaved by the enzyme. Polymers I-IV form stable specific complexes with EcoRII endonuclease (Fig. 4 and Table). The binding curves are hyperbolic for all the substrates, which makes it possible to conclude that the protein binds to DNA duplexes non-cooperatively [18].

Our substrates are formed by polynucleotides of different length and contain many EcoRII recognition sites. Thereby several molecules of the enzyme may bind to one molecule of a DNA duplex and the stoichiometry of this binding is not known. Therefore we cannot define exactly the equilibrium association constants for enzyme-substrate complexes. For this reason, as a parameter for the thermodynamic stability of endonuclease EcoRII complexes with DNA duplexes I-V we used the magnitude $K' = (E_o)^{-1}_{0.5}$, where $(E_o)_{0.5}$ is the overall enzyme concentration in a mixture with the substrate (C_N 125 nm), at which R = 0.5. According to their thermodynamic stability EcoRII complexes with polymers I-V may be arranged as I>III>IV>II>>V (Fig. 4

Table
The characteristics of endonuclease EcoRII complexes with polymers I-V*

Polymer	Stability of the complex, K', M^{-1}	Dissociation rate constant min^{-1}	Half-life period ($T_{\frac{1}{2}}$), min	Rate** of substrate cleavage
I	$(1.6\pm0.5)\cdot10^{9}$	$(3.5\pm0.1)\cdot10^{-3}$	200±2	5.8
II	$(3.6\pm0.5)\cdot10^{8}$	$(8.7\pm0.1)\cdot10^{-3}$	80±2	2.9
		0.33±0.01***	2±1	
III	$(1.2\pm0.5)\cdot10^{9}$	$(1.9\pm0.1)\cdot10^{-3}$	360±2	7.2
IV	$(8.3\pm0.5)\cdot10^{8}$	$(1.1\pm0.1)\cdot10^{-3}$	640±2	0.1
V	$(1.5\pm0.3)\cdot10^{7}$	>0.7	<1	-

* Enzyme binding to the substrates and dissociation of the complexes were studied in buffer 2 at 37°C.
** The number of phosphodiester bond (pmol) cleaved during the first 10 minutes of the reaction. Conditions are given in "Materials and Methods".
*** Data for the two types of complexes (Fig. 6 and text)

and Table). The differences in the K' values for substrates I-IV do not go beyond one order of magnitude. In the case of polymer V the K' value, characterizing non-specific interaction, is by two orders less than the K' for polymer I, containing natural EcoRII recognition sites.

To obtain kinetic parameters of the complexes of EcoRII endonuclease with polymers I-V we studied their dissociation in the presence of calf thymus DNA which contains natural EcoRII sites and operates as a competitive agent (Fig. 5). We

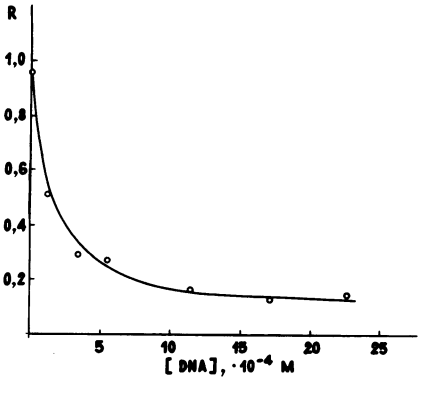

Fig. 5. Dependence of polymer I binding to endonuclease EcoRII on the concentration of competitive calf thymus DNA. Buffer 2, C_N 125 nM, 8 nM of EcoRII endonuclease, 37°C.

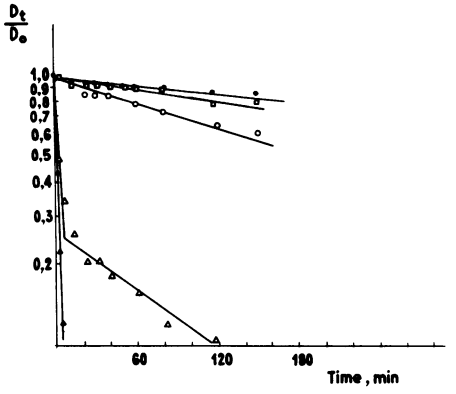

Fig. 6. Dissociation of complexes of polymers I (o), II (△), III (□), IV (●) and V (▲) with endonuclease EcoRII in the presence of calf thymus DNA (1.1 mM per nucleotide residue) in buffer 2 at 37°C. C_N 125 nM, 8 nM (for polymers I-IV) or 28 nM (for polymer V) of EcoRII endonuclease. D_o is the initial amount of complexes, D_t is the amount of complexes at different time intervals.

choose conditions (see "Materials and Methods" and Figs 4,5) at which complexation does not depend on the concentrations of the enzyme and competitive DNA. The data on dissociation kinetics for EcoRII complexes with polymers I-V are given in Fig. 6. The dissociation rate constants were calculated by the equation for the first order reaction [19], half-like periods ($T_{\frac{1}{2}}$, Table) were determined from the kinetic curves. A non-specific complex formed by endonuclease EcoRII with polymer V is noted for its extreme fast dissociation. Its $T_{\frac{1}{2}}$ is less than 1 min. The dissociation curve of the EcoRII complex with polymer II has a break (Fig. 6) thus showing the presence of two types of complexes with $T_{\frac{1}{2}}$ of 50 and 2 minutes. Apparently the less stable complex of the enzyme with polymer II may be considered as non-specific since its $T_{\frac{1}{2}}$ is very close to that for such complex with polymer V which contains no recognition sites. The complex with $T_{\frac{1}{2}}$ 50 min may be considered as a specific one. By using the dissociation curve, we assayed the content of long-lived complex as 26% and that of short-lived one as 74%. The $T_{\frac{1}{2}}$ values for the specific complexes are arranged as IV>III>I>>II. Consequently, the substitution of br^5dU for the dT residue and of m^5dC for the 5'-terminal dC residue in the dT-containing strand does not accelerate dissociation of the complex. Conversely, the absence of the 5-methyl group in the dT residue of the central AT-pair of the recognition site (in the case of polymer II) significantly increases the rate of dissociation of the complex.

It is noteworthy that dissociation of the EcoRII complex with polymers III and IV proceeds more slowly than that of the complex with polymer I (see Table). Since polymers I and III are cleaved in a processive mode, the lower dissociation rate of the complex formed by polymer III and the enzyme may be conducive to multiple cleavage of the substrate in a single enzyme-substrate binding event. This may be one of the reasons for a high rate of hydrolysis of polymer III compared with polymer I and for the discrepancy between our results and those of Petruska and Horn [4].

DISCUSSION

The modifications used in this work are located at position of some dC and dT residues and do not prevent formation of complementary pairs. Also the atoms H and CH_3-groups at position 5 of pyrimidine nucleotides in the case of the B-form DNA structure, common for water solutions, are readily accessible to molecules of water [20] and, consequently, can effectively interact with the protein. On the other hand, the base modifications used in this work probably cannot cause significant alteractions in the DNA structure. One of the proofs is that the circular dichroism spectrum of a complementary complex formed by 9A and 9M is very close to the earlier studied one [7] of a complex formed by 9A and 9T. Therefore our data on the effect of base modifications on the binding and cleavage of the substrate by endonuclease EcoRII attest firstly, to a hydrophobic contact of the enzyme with the CH_3-group of the dT residue of recognition site. Such contact is disturbed if the hydrogen atom is substituted for the methyl group. This is the most likely reason for a reduced stability of the complex of polymer II with the enzyme and for a decreased rate of hydrolysis of this substrate. At the same time a slight change of the complex stability and cleavage rate as br^5dU is substituted for the dT residue is quite consistent with the similar sizes of bromine atom and methyl group. Secondly, an abrupt slow-down in EcoRII cleavage of polymer IV as compared with non-modified substrate I testifies to a contact (or at least to close proximity) of the protein with atom H^5 in the 5'-terminal dC residue of the

dT-containing strand of the recognition site. The investigated enzyme must be also in contact with H^5 atoms of the two dC residues contiguous to the central AT-pair of the recognition site, since the CH_3-groups substitute for these very atoms as a result of DNA modification by methylase EcoRII which prohibits subsequent cleavage of the substrate by endonuclease EcoRII [2]. These protein-nucleic contacts are located in the major groove of the DNA double helix. According to X-ray diffraction data for the EcoRI endonuclease-substrate complex [21], all the contacts of this enzyme with DNA bases capable of ensuring recognition of the nucleotide sequence are located only in the major groove of the recognition site.

Our investigation of the cleavage of polymers II-IV by endonuclease EcoRII showed that modification of bases in one of the two strands of the recognition site (in the dT-containing strand) has an equal effect on the cleavage of both strands of the substrate. It follows from the very slow rate of cleavage of both strands of the recognition sites in polymer IV (even signle-strand cleavages which are possible for EcoRII endonuclease [8,22] are not observed for this substrate) and from the fact that there is no preferential cleavage of unmodified or modified strands in polymers II and III. These results enables us to conclude that recognition of the exposed groups of DNA bases and hydrolysis of both strands of the EcoRII recognition site take place within a single complex. This agrees with our conclusion on the interaction of EcoRII endonuclease with both strands of the DNA recognition site [8,22]. Possibly both strands of this site (the dT- and dA-containing ones) are also involved in the recognition of the substrate. This is confirmed by the ability of endonuclease EcoRII to recognize and cleave the substrate under both possible orientations of the central AT-pair of the recognition site with respect to the bound enzyme molecule [15] and also by the specific cleavage by this enzyme of DNA duplexes at the ...↓C-C-T-G-G-... and ...-G-G-T-C-C↑... sites [8].

...↓C-C-A-G-G-...
...-G-G-A-C-C↑...

Studying the complexation of the endonuclease EcoRII with polymers I-V revealed that this enzyme recognizes the substrate

when binding to it. This is testified by the following evidences. First, the enzyme forms much more stable and much more long-lived complexes with DNA duplexes I-IV than with polymer V which does not contain EcoRII recognition sites. Second, a significant decrease in the stability of the complex occured when the AU-pair was substituted for the central AT-pair of the recognition site, which quite correlates with the decrease of the substrate cleavage rate. Third, the specificity of EcoRII binding to the substrate is also confirmed by the fact that substitution of pyrophosphate [23] or phosphoamide [22] bonds for endonuclease EcoRII - scissile phosphodiester bonds completely blocks the substrate cleavage and does not prevent its binding by the enzyme, i.e. makes the enzyme-substrate complex unproductive.

It is interesting to note that stable specific complex formed by EcoRII endonuclease with polymer IV is unproductive either. But in this case methylation of one of dC residues prevents the cleavage both of modified and unmodified strand of the recognition site. On the other hand, our enzyme can catalyze single-strand breaks if the cleavage of another strand is blocked by one of above mentioned modifications of the scissile phosphodiester bond [22]. On our mind, there is no discrepancy between these two results because the enzyme can respond differently to modification of DNA bases and internucleotide bonds. So different modes of specific binding of EcoRII endonuclease to modified substrates may be suggested.

The obtained results showed that tight binding and effective cleavage do not always go hand-in-hand. Consequently, the specificity of EcoRII endonuclease action, as in the case of EcoRI [24], is not confined only to a tight binding to a natural substrate. A compulsory condition for DNA cleavage is the realization of all (or almost all) essential protein-nucleic acid contacts, some of which may be lacked when the substrate is modified. Additional proof thereof is that endonuclease EcoRII, capable to some extent of non-specific binding to DNA (Fig. 4), cleaves the substrate only specifically (Fig. 2 and [8,15]).

In the previous papers [15,22] we suggested that the structure of the endonuclease EcoRII-substrate complex may corres-

pond to a "symmetric model" described in the literature [25], according to which two identical subunits of the protein are positioned symmetrically on two DNA strands with respect to the rotational symmetry of the recognized sequence. Possibly, two protein subunits, as in the case of EcoRI endonuclease [21,26], operate cooperatively in the specific binding of the substrate. One may suggest that the same cooperativity is also manifest in the formation of two hydrolytical active centers near the two scissile bonds. The fact that methylation of 5'-terminal dC in the dT-containing strand of the EcoRII recognition site does not prevent enzyme-substrate binding but strongly inhibits the cleavage of both DNA strands is in favor of this suggestion. Since the investigated enzyme can catalyse single-strand cleavages [8,22] one may also suppose that being formed, these hydrolytical active centers operate independently of each other in cleavage of internucleotide bonds.

Proceeding from the "symmetric model" of the enzyme-substrate complex, it would be of particular interest to consider endonuclease EcoRII interaction with the central AT-pair distorting the rotational symmetry of the DNA recognition site. We found that the enzyme recognizes the most asymmetric element of this pair - the methyl group of the dT residue. Yet a change in the orientation of this AT-pair within the enzyme-substrate complex has no effect on the recognition and cleavage of the substrate by endonuclease EcoRII [15]. All these results considered, we may suggest that this enzyme can sufficiently readily attune its structure to the orientation of the "asymmetrical" AT-pair in the bound substrate.

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