Cleavage of synthetic substrates containing non-nucleotide inserts by restriction endonucleases. Change in the cleavage specificity of endonuclease *SsoII*

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ABSTRACT

A study was made of the interaction between restriction endonucleases recognizing CCNGG (*SsoII* and *ScrFI*) or CC^A/TGG (*MvaI* and *EcoRII*) DNA sequences and a set of synthetic substrates containing 1,3-propanediol, 1,2-dideoxy-D-ribofuranose or 9-[1'-hydroxy-2'-(hydroxymethyl)ethoxy] methylguanine (glG) residues replacing either one of the central nucleosides or dG residues in the recognition site. The non-nucleotide inserts (except for glG) introduced into the recognition site both increase the efficiency of *SsoII* and change its specificity. A cleavage at the noncanonical position takes place, in some cases in addition to the correct ones. Noncanonical hydrolysis by *SsoII* occurs at the phosphodiester bond adjacent to the point of modification towards the 5'-end. With the guanine base returned (the substrate with glG), the correct cleavage position is restored. *ScrFI* specifically cleaves all the modified substrates. DNA duplexes with non-nucleotide inserts (except for the glG-containing duplex) are resistant to hydrolysis by *MvaI* and *EcoRII*. Prompted by the data obtained we discuss the peculiarities of recognition by restriction endonucleases of 5-membered DNA sequences which have completely or partially degenerated central base pairs. It is suggested that *SsoII* forms a complex with DNA in an 'open' form.

INTRODUCTION

In the last few years a great number of type II restriction enzymes have been isolated. They have been widely used in a variety of molecular biology fields. However the mechanism of their interaction with DNA is still unknown. This molecular mechanism is becoming better understood through studies on the interaction between these enzymes and modified oligodeoxyribonucleotide duplexes [1–6]. Such a study has demonstrated that restriction enzymes can recognize and cleave many modified substrates. In the majority of cases a decrease

in hydrolysis efficiency or complete inhibition of cleavage was observed [1–4], though introduction of some modifications resulted in a higher activity of some endonucleases, a representative example being *SsoII* endonuclease [5,6]. It should be noted that in no cases did a change of the positions of cleavage at the recognition sites take place. Hitherto only a partial decrease in the specificity of recognition of type II restriction endonucleases (so-called relaxed, or 'star', specificity) was observed when conditions of the enzymatic reaction were modified [7–9].

This paper deals with the study of the cleavage by type II restriction endonucleases of analogs of substrates with one of the nucleoside residues in the recognition site replaced by 1,3-propanediol, 1,2-dideoxy-D-ribofuranose or 9-[1'-hydroxy-2'-(hydroxymethyl)ethoxy] methylguanine. The substitutions were made to elucidate the role of some heterocyclic bases and the sugar-phosphate backbone in the specific interaction of these enzymes with DNA [9].

Restriction endonucleases, recognizing the 5-membered sequence with a partially (*EcoRII* [10] and *MvaI* [11]) or completely (*ScrFI* [12] and *SsoII* [13]) degenerated central base pair were investigated:

(here N indicates A, T or G, C; W indicates A or T and the arrows indicate the position of the cleavage sites). *SsoII* and *ScrFI* as well as *EcoRII* and *MvaI* are isoschizomers which cleave the corresponding recognition site in different positions. Substrate analogs containing abasic nucleoside residues in the central position of recognition site were of special interest since we were interested in defining the structural elements of the DNA which

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could be recognized by restriction endonucleases in the degenerated position. On the basis of the results obtained a comparative analysis was made of the mechanism of action of *SsoII*, *ScrFI*, *EcoRII* and *MvaI* endonucleases.

MATERIALS AND METHODS

DNA duplexes

14-mer nonmodified oligodeoxyribonucleotide constituents of DNA duplexes I–X were synthesized according to the standard phosphoramidite technique on a Cyclone synthesizer (Biosearch, U.S.A.) and were kindly donated by Dr. Oretskaya. Modified strands of DNA duplexes II–VII and IX–X were constructed by Dr. Volkov and Dr. Romanova as described earlier [14–16]. $5'$ - 32 P-labelling of the oligodeoxyribonucleotides were carried out using T4 polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The $5'$ -terminal label was introduced alternatively in one of the strands of DNA duplexes I–X.

Table 1. Cleavage of synthetic DNA duplexes I–X by restriction endonucleases

No	DNA duplex ^a	Relative cleavage % ^b			
		<i>SsoII</i>	<i>ScrFI</i>	<i>MvaI</i>	<i>EcoRII</i>
I	5' <u>ACCTACCTGGTGGT</u> 3'	100	100	100	100
	3' <u>TGGATGGACCA</u> 5'	100	100	100	100
II	...C-C-Prd-G-G...	220 ^c	51	0 ^e	0 ^e
	...G-G-A-C-C...	118	100	1 ^e	0 ^e
III	...C-C-T-G-G...	211	106	86 ^e	0 ^e
	...G-G-Prd-C-C...	136 ^c	17	3 ^e	0 ^e
IV	...C-C-ddR-G-G...	237 ^c	28	5	0
	...G-G-A-C-C...	158	91	1	0
V	...C-C-T-ddR-G...	195 ^d	79	9	0
	...G-G-A-C-C...	68	67	0	0
VI	...C-C-T-G-G...	33	53	19	0
	...ddR-G-A-C-C...	163 ^d	18	0	0
VII	...C-C-T-glG-G...	37	89	64	4
	...G-G-A-C-C...	86	36	64	3
VIII	5' <u>GCCAACTGGCTCT</u> 3'	100	100	100	100
	3' <u>CGGTTGGACCGACA</u> 5'	100	100	100	100
IX	...C-C-Prd-C-C...	321 ^c	91	1	0
	...G-G-A-C-C...	174	71	5	0
X	...C-C-T-G-G...	207	85	30	0
	...G-G-Prd-C-C...	211 ^c	65	13	0

Prd, 1,3-propanediol; ddR, 1,2-dideoxy-D-ribofuranose; glG, 9-[1'-hydroxy-2'-(hydroxymethyl)ethoxy]methylguanine

a. Index d (deoxy) is omitted. In duplexes II–VII and IX, X only modified fragments of the molecule are shown. The other parts of substrates II–VII and IX, X are identical with starting compounds I and VIII, respectively.

b. The ratio of the cleavage % of individual strands of modified DNA duplexes II–VII and IX, X to cleavage % of the corresponding strands of the starting duplexes I and VIII, respectively, at the same conditions. The data in the upper and lower lines relate to 'upper' and 'lower' strands of DNA duplexes.

c. Relative cleavage % of modified strands of duplexes II–IV, IX and X at canonical and noncanonical sites.

d. Relative cleavage % of modified strands of duplexes V and VI at noncanonical site.

e. These values had been determined previously [17].

Enzymes

SsoII endonuclease (10000 units/ml) was isolated from *Shigella sonnei* strain 47 as described in [13]. *ScrFI* endonuclease (10000 units/ml) was a commercial preparation from Serva (F.R.G.). *EcoRII* endonuclease (400 units/ml) was purchased from NPO Biolar (Latvia). *MvaI* endonuclease (60000 units/ml) and T4 polynucleotide kinase were purchased from NPO Ferment (Lithuania). The restriction endonuclease activity unit was defined as the amount of enzyme able to digest completely 1 mg of λ phage DNA at 37°C in 1 h.

Hydrolysis of DNA duplexes by restriction endonucleases

The ^{32}P -labelled substrates I–X (concentration per duplex C_D 3.5×10^{-7} M) were incubated at 10°C for 2 h with 10 units of *SsoII*, 10 units of *ScrFI*, 0.04 units of *EcoRII* or 60 units of *MvaI* in 10 ml of 10 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 10 mM MgCl_2 , 1 mM dithioerythritol (*SsoII*); 10 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 5 mM MgCl_2 , 1 mM mercaptoethanol (*ScrFI*); 40 mM Tris-HCl buffer, pH 7.6, 50 mM NaCl, 5 mM MgCl_2 , 7 mM dithiothreitol (*EcoRII*); 10 mM Tris-HCl buffer, pH 8.5, 15 mM MgCl_2 , 150 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml of bovine serum albumin (*MvaI*).

Analysis of cleavage products

Enzymatic reactions were stopped by heating at 95°C for 2–4 min. The products of cleavage of ^{32}P -labelled DNA duplexes I–X were analyzed in 20% polyacrylamide gel containing 7M urea. After autoradiography the radioactivity of the gel slices was determined by Cherenkov counting. The cleavage percent was defined for each strand of the substrate separately as the ratio of the radioactivity of the hydrolysis product to the total radioactivity of the product and uncleaved substrate.

The length of products of cleavage of DNA duplexes V and VI was determined by ion-pair reversed-phase HPLC on a Waters chromatograph (U.S.A.); column 4×250 mm with Diasorb C-16T, 7.5 mm (ELSIKO, Russia). Oligonucleotide fragments were eluted with a logarithmic gradient of 5–40% acetonitrile in 48 mM potassium phosphate buffer (pH 7.0) with 2 mM tetrabutylammonium phosphate.

RESULTS AND DISCUSSION

Substrate analogs design

We used the novel types of modified oligonucleotide duplexes (Table 1). The first category are analogs of the *SsoII*, *ScrFI*, *EcoRII* and *MvaI* substrates with the heterocyclic base removed but with the sugar-phosphate backbone partially or completely preserved. Thus, the modified DNA contains propylene bridges (duplexes II, III, IX and X) or 1,2-dideoxy-D-ribofuranose residues (duplexes IV–VI) instead of one of the nucleoside residues of the recognition site. 1,2-dideoxy-D-ribofuranose substituted for 2-deoxy-D-ribofuranose prevents the destruction of modified oligonucleotide constituents of DNA duplexes IV–VI during synthesis. The second category are analogs of substrates with the sugar moiety broken but with the heterocyclic base preserved. In this work the glyceroguanine derivative 9-[1'-hydroxy-2'-(hydroxymethyl)ethoxy]methylguanine (duplex VII) was used. Replacement of T, A or G residues by 1,2-dideoxy-D-ribofuranose or 1,3-propanediol residue makes it possible to reveal the role of thymine, adenine or guanine residues in protein-nucleic acid interactions. The proposed DNA

duplexes modified in such a way (Table 1) have advantages over substrate analogs with some nucleoside residues completely removed [17]. Replacement of 1,2-dideoxy-D-ribofuranose residue by 1,3-propanediol allows one to distinguish the role of the sugar moieties of the substrate in the interaction with protein. All of the above modifications were introduced within the recognition site of these enzymes which was located in the central part of 14-mer DNA duplex I (Table 1).

The modified DNA duplexes (II–IV, VI and VII) have been studied previously by UV-spectroscopy and CD [15, 16]. It was shown that introducing 1,3-propanediol or 1,2-dideoxy-D-ribofuranose residues leads to a 19°C destabilization of DNA duplexes II–IV and VI. This is accounted for by the disturbance of the Watson–Crick interactions and the increasing conformational mobility of groups of atoms adjacent to the modified site. The introduction of glG residue into 14-mer DNA duplex I lowers the melting temperature by 14°C. The melting curve of this modified substrate (VII) is of a more cooperative character than those of duplexes II–IV and VI. It is similar to that of a nonmodified substrate [15]. Analysis of CD spectra of the modified DNA shows that there are only minor conformation distortions of double helix induced by single modifications [15, 16]. Local structural alterations in DNA may occur at the site of modification. Of all non-nucleotide inserts we introduced into DNA glG affects the double helix structure to the least extent. Cleavage of substrate analogs II–X by restriction endonucleases was performed at 10°C under conditions of duplex stability.

Cleavage of modified DNA by *SsoII* and *ScrFI* restriction endonucleases. Change in the cleavage specificity of *SsoII*

The isoschizomeric enzymes *SsoII* and *ScrFI*, which have the recognition site completely degenerated at the central base pair, cleave all the modified DNA duplexes (Table 1, Figs. 1,2). Remarkably, in the case of *SsoII* non-nucleotide analogs within its recognition site affect hydrolysis in an unusual way. Replacement of A or T residues at the central position of the recognition site by a propylene bridge or 1,2-dideoxy-D-ribofuranose results in a considerable increase in the efficiency of *SsoII* cleavage of each of the strands of DNA duplexes II–IV, IX and X and also produces the noncanonical breaks side by side

with canonical ones (Table 1, Figs. 1, 3). It is noteworthy that for each of these substrates one novel site of cleavage is formed. It is adjacent to the non-nucleotide insert towards the 5'-end. Duplexes II and IX as well as III and X, which possess different nucleotide sequences flanking the modified *SsoII* site, reveal similar substrate properties. Replacement of one of the G residues within the recognition site by 1,2-dideoxy-D-ribofuranose leads to a change in the site of *SsoII* hydrolysis of the modified strands of duplexes V and VI (Figs. 1,3,4). The efficiency of cleavage of the modified strand increases while that of the nonmodified strand decreases (Table 1). In duplexes V and VI as well as II–IV, IX and X a novel site of digestion, a cleavable phosphodiester bond, is located in the close vicinity of the introduced non-nucleotide insert (towards the 5'-end) (Figs.3,4).

The ability of *SsoII* to effectively and specifically cleave DNA duplexes II–IV, IX and X suggests that this endonuclease can recognize such a 'faulty' site and that at least at the binding step the enzyme does not interact with each of the bases of the central degenerated base pair of the *SsoII* recognition site. Apparently *SsoII* is able to function without having the intact sugar moiety in the central nucleoside residues. This is confirmed by the fact that no differences were observed in the hydrolysis of substrates with a propylene bridge (II) or 1,2-dideoxy-D-ribofuranose (IV) substituted for a T residue (Table 1). At the same time the correct cleavage of substrates II–IV, IX and X is accompanied by noncanonical hydrolysis of the modified strand of these duplexes. We think that a noncanonical break is stimulated by a structural distortion of DNA at the site of modification due to removal of the heterocyclic base. The fact that *SsoII* cleaves the noncanonical phosphodiester bonds in duplexes II–IV, IX and X just near the abasic site (Figs.1,3) supports this suggestion. In duplexes V and VI the noncanonical break is located in the same position (Figs. 1,3,4). However, there is a fundamental difference in the cleavage behaviour of substrates V and VI as compared with duplexes II–IV, IX and X. In this case the correct cleavage of modified strands does not take place at all. This may indicate that *SsoII* must interact with the inner and outer guanine residues to ensure specific hydrolysis. The substrate properties of duplex VII, which in contrast to substrate V had the guanine intact but the sugar moiety of the inner G residue broken, were also studied. A

Figure 1. Autoradiograph of the gel-electrophoretic separation of oligonucleotide components in a *SsoII* endonuclease cleavage of 'lower' (a) and 'upper' (b) strands of duplex I and modified strands of duplexes II–VII. The length of the oligonucleotides is shown at the right.

Figure 2. Autoradiograph of the gel-electrophoretic separation of oligonucleotide components in a *ScrFI* endonuclease cleavage of duplexes I–III. The ^{32}P -label is in the 'upper' strand (a) or in the 'lower' strand (b). The reaction time (min) is shown at the top. XC, xylene cyanol; BPB, bromphenol blue. The length of the oligonucleotides is shown at the right.

Figure 3. Scheme of canonical (filled arrows) and noncanonical (open arrows) cleavage sites of duplexes I–X by *SsoII* endonuclease.

considerable decrease in the rate of cleavage of the modified strand of duplex VII was observed (Table I). Remarkably, substrate VII is cleaved by *SsoII* at the canonical positions (Figs.1,3). This proves that the above heterocyclic base is extremely important for providing specific interactions of *SsoII* with the DNA double helix.

It is noteworthy that the ability to change the cleavage specificity and cut the substrate more effectively have been revealed only in the case of *SsoII* hydrolysis of substrates with non-nucleotide inserts. This phenomenon is not observed when duplexes II–VI, IX and X are cleaved by *ScrFI*, *EcoRII* and *MvaI* (see below), and when DNA duplexes with 1,3-butanediol residues in the *EcoRI* recognition site are cleaved by *EcoRI* [18]. What is the reason for such behaviour? Earlier we observed an increase in the efficiency of *SsoII* hydrolysis of the substrate containing a hybrid rU·dA nucleotide pair at the central position of the recognition site [5]. It was proposed [5] that the DNA transition state in the *SsoII*-substrate complex resembles A-DNA and that an rU residue introduced into the *SsoII* site contributes to the substrate's A-like conformational change. However, the efficiency of *SsoII* action was observed to increase also when a mismatched T·T pair [6] or non-nucleotide inserts (Table 1) were introduced into the *SsoII* recognition site. This suggests that DNA in the complex with *SsoII* undergoes more considerable

Figure 4. The ion-pair reversed-phase HPLC separation of oligonucleotide components following *SsoII* endonuclease cleavage of duplexes I (a), V (b) and VI (c).

conformational disturbance than merely a transition into an A-like form. The introduction of a mismatched base pair or a non-nucleotide insert may result in an increase in the conformation mobility of the DNA double helix at the site of modification followed by a decrease in the energy barrier of DNA conformational change and an augmentation of the enzymatic reaction rate. Besides, the site of DNA duplex cleavage by *SsoII* moves into the region of the introduced non-nucleotide insert. The cleavage of the substrates with non-nucleotide inserts by *R·SsoII* at the novel phosphodiester bonds is not, probably, due to the same catalytic functions of the enzyme as the cleavage at the canonical positions of the recognition site. Involving in the catalysis the amino acids but not those at the active site of the protein is rather possible.

It should also be stressed that different strands of the same duplex (substrates II–VII, IX and X) exhibit different efficiency of cleavage by *SsoII*. Different cleavage rates of separate strands have been observed earlier for the *SsoII* hydrolysis of DNA duplexes containing uridine or 5-fluorodeoxyuridine residues in the recognition site [5]. The study of the *SsoII* hydrolysis of substrates with non-cleavable phosphoramidate bond replacing a cleavable phosphodiester bond did not reveal any influence of the modification in one strand on the hydrolysis of the second [19]. Hence *SsoII* enzyme digests different strands of such modified DNA duplexes in an independent manner. From all the data it may be suggested that DNA is unwound in the complex with *SsoII* forming a so-called 'open complex' similar to those which DNA forms with other DNA-binding enzymes.

Introducing modifications into the *ScrFI* recognition site lowers the efficiency of cleavage of DNA duplexes II–VII, IX and X by *ScrFI*, an isoschizomer of *SsoII* (Table 1, Fig.2). However, *ScrFI* inhibits hydrolysis less than the *EcoRII* and *MvaI* enzymes (see below). Besides, in contrast to *SsoII*, no changes in the specificity of the cleavage of these substrates by the *ScrFI* enzyme were observed. The rate of hydrolysis of modified duplexes by *ScrFI* endonuclease depends on the location of the non-nucleotide insert. Substitution for one of the central nucleosides of the recognition site leads in the majority of cases to a lower efficiency of hydrolysis of the modified strand, but the non-modified strand is cleaved with its initial efficiency (Table 1). Substitution of 1,2-dideoxy-D-ribofuranose for one of the inner or outer G residues slightly inhibits the hydrolysis of both strands of the DNA duplexes V and VI. No fundamental differences were observed between the substrate properties of DNA duplexes V with the guanine residue removed and VII with the guanine residue restored. It may be supposed that the A and T residues in the central degenerated position of the recognition site and one of the inner G residues are not important for DNA duplex interaction with *ScrFI* and that the minor effects we observed are due to substrate conformational changes when non-nucleotide inserts are introduced.

Decrease in the efficiency of cleavage of modified substrates by restriction endonucleases *MvaI* and *EcoRII*

Replacement of A or T residues in the recognition site by 1,3-propanediol or 1,2-dideoxy-D-ribofuranose residues (duplexes II–IV, IX and X) inhibits the cleavage of both strands of the duplexes by *EcoRII* and *MvaI* (except for the nonmodified strand of substrate II which is digested by *MvaI* effectively) (Table 1). *EcoRII* does not cleave either substrates with the guanine residues removed (substrates V and VI), and *MvaI* hydrolyses only the non-modified strand of V and VI, but less effectively.

If one of the removed guanine residues is restored by introducing the deoxyguanosine acyclic analog (substrate VII), enzyme activity will be partially restored (more so for *MvaI*). All these data testify that the *EcoRII* and *MvaI* enzymes interact with each of the bases of the central 'asymmetrical' A·T-pair as well as with the outer and inner guanine residues of the recognition site. Similar results were obtained with *EcoRI* [18]. Replacement of any nucleoside residue of this enzyme recognition site by 1,3-butanediol residue blocks *EcoRI* action. It should be noted as well that the DNA duplex VII containing *glG* is cleaved by *EcoRII* but less effectively. The guanine base can probably freely rotate owing to the acyclic sugar residue. This may prevent complementary interactions in the *glG*·C-pair and some protein-nucleic acid contacts. Thus, to function effectively *EcoRII* needs the deoxyribose residue to be intact in the inner deoxyguanosine.

CONCLUSION

A fundamental difference was demonstrated in the interaction of two groups of restriction endonucleases: *SsoII* and *ScrFI* enzymes and *MvaI* and *EcoRII* enzymes with substrate analogs containing non-nucleotide inserts. The two groups show different tolerance towards the degeneracy of the central base pair. Restriction endonucleases of the first group, recognizing the CCNGG-sequence, can effectively cleave DNA duplexes containing an abasic site in the centre of the recognition sequence or replacing one of the G residues. Restriction endonucleases of the second group recognizing only a partially degenerated site do not cleave such substrates. In addition, *SsoII* is shown to cleave modified strands of these substrates at noncanonical sites as well. The substitution of non-nucleotide analogs for degenerated nucleotide pairs stimulates a partial change in the cleavage specificity of this enzyme. When one of G residues is replaced, the *SsoII* cleavage specificity is altered completely. The results obtained contribute to the data on the exceptional variety of mechanisms of action of different type II restriction endonucleases.

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