The interaction of DNA duplexes containing 2-aminopurine with restriction endonucleases *EcoRII* and *SsoII*

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ABSTRACT

Oligonucleotides containing 2-aminopurine (2-AP) in place of G or A in the recognition site of *EcoRII* (CCT/AGG) or *SsoII* (CCNGG) restriction endonucleases have been synthesized in order to investigate the specific interaction of DNA with these enzymes. Physicochemical properties (CD spectra and melting behaviour) have shown that DNA duplexes containing 2-aminopurine exist largely in a stable B-like form. 2-Aminopurine base paired with cytidine, however, essentially influences the helix structure. The presence of a 2-AP·C mismatch strongly reduces the stability of the duplexes in comparison with the natural double strand, indicated by a biphasic melting behaviour. *SsoII* restriction endonuclease recognizes and cleaves the modified substrate with a 2-AP·T mismatch in the centre of the recognition site, but it does not cleave the duplexes containing 2-aminopurine in place of inner and outer G, or both. *EcoRII* restriction endonuclease does not cleave duplexes containing 2-aminopurine at all. The two-substrate mechanism of *EcoRII*–DNA interaction, however, allows hydrolysis of the duplex containing 2-aminopurine in place of adenine in the presence of the canonical substrate.

INTRODUCTION

Type II restriction endonucleases are used as reagents in a wide variety of biological and medical fields. Because they recognize DNA with such high specificity, the study of these enzymes themselves can improve our understanding of DNA–protein interactions. In addition to X-ray crystallography and site-directed mutagenesis, the use of modified oligodeoxyribonucleotide duplexes is an important tool in such studies, aimed at elucidating the molecular mechanisms of the interactions between enzymes and DNA (1–5).

We are interested in the mechanism of recognition and cleavage of DNA by the *EcoRII* and *SsoII* restriction endonucleases. These

enzymes recognize a related 5 bp sequence in duplex DNA. For *EcoRII* the central base pair is partially degenerated and for *SsoII* the central base pair is completely degenerated:

It appears that the mechanism of *EcoRII*–DNA interaction is more complex than that of most type II restriction enzymes (6–8). *EcoRII* requires two recognition sites for catalytic function (9,10). Using modified substrate analogues we have shown that cleavage of one of the recognition sites depends on cleavage of the other (9). The formation of a four-stranded recognition site for the *EcoRII*–substrate active complex was suggested.

By studying the cleavage of synthetic substrates containing non-nucleotide inserts, such as a propylene bridge or 1,2-di-deoxy-D-ribofuranose, in the *SsoII* restriction endonuclease recognition site we have shown that the cleavage and specificity of *SsoII* is altered. It was suggested that in the catalytically active *SsoII*–substrate complex DNA is transformed to a single-stranded state at the cleavage site ('open' form) (11).

To investigate the two-substrate mechanism of *EcoRII*–DNA interaction and the possibility of specific binding of *SsoII* to DNA in an 'open' form we have studied a number of oligonucleotides containing 2-aminopurine (2-AP) at different positions in the target sequence as substrates for both enzymes. In order to realize that, we developed an efficient synthesis of 2-aminopurine-2'-deoxyriboside and its fully protected phosphoramidite building block suitable for incorporation into oligodeoxyribonucleotides (12).

MATERIALS AND METHODS

Phosphodiesterase from *Crotalus durissus* (0.003 U/ml) and calf intestine alkaline phosphatase (22 U/ml) were obtained from Boehringer Mannheim. *EcoRII* (EC 3.1.21.4.) (0.4 U/μl) was

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purchased from NPO Biolar (Latvia). *SsoII* (EC 3.1.21.4.) (10 U/μl) was produced and purified as described (13). T4 polynucleotide kinase (EC 2.7.1.78.) was purchased from NPO Ferment (Lithuania). One unit of restriction endonuclease activity was defined as the amount of the enzyme which completely digests 1 μg phage λ DNA at 37°C in 1 h.

UV spectra were measured in H₂O (pH 7.0) using a Shimadzu 160 A UV/VIS spectrometer. HPLC was performed using a LC6A gradient system (Shimadzu) and an ICI/GAT gradient system. Buffers were as follows: A, 50% 0.1 M triethylammonium acetate (TEAA)/50% CH₃CN, pH 7; B, 98% 0.1 M TEAA/2% CH₃CN, pH 7; C, 0.04 M KH₂PO₄, pH 4.2; D, 0.04 M Tris-HCl, 0.04 M MgCl₂, pH 8.9; E, 0.015 M sodium citrate, pH 7.25; F, 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 7 mM dithiothreitol; G, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol.

Phosphoramidite building block

The 5'-*O*-dimethoxytritylated *N*²-isobutyryl-protected phosphoramidite of the modified nucleoside was prepared using chloro-(2-cyanoethoxy)-diisopropylaminophosphine according to standard procedures (14–16).

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized by the phosphoramidite method (17) on an automated DNA synthesizer (Gene Assembler Plus; Pharmacia) with chain assembly on the 0.2 mmol scale. The modified phosphoramidite building block was dissolved in dry acetonitrile at a concentration of 0.2 M and filtered through 0.45 mmol Teflon filters prior to use. Syntheses were performed 'trityl on', deblocking was performed using 32% aqueous ammonia at 50°C overnight. Dimethoxytrityl-containing oligonucleotides were purified by HPLC using an RP 18 column (250 × 13 mm, 3 ml/min; Machery and Nagel) with buffer A and a linear gradient of 10–50% buffer B over 40 min. Oligonucleotides containing the dimethoxytrityl group eluted after 25 min. Product-containing fractions were evaporated to dryness *in vacuo*, redissolved in 80% acetic acid (200 μl) and allowed to react at room temperature for 40 min. Acetic acid was removed by evaporation *in vacuo*, followed by co-evaporation with water (2 × 200 μl). The resulting product was dissolved in 500 μl water and extracted with ethyl acetate (3 × 100 μl). The aqueous layer was evaporated to dryness and the oligomers stored at –20°C. The yield was about 6 OD₂₆₀ units/oligonucleotide.

Nucleoside composition analysis

Each oligonucleotide (0.2 OD₂₆₀ units) was dissolved in 6 μl buffer D. Snake venom phosphodiesterase (3 μl) and alkaline phosphatase (1 μl) were added and the mixtures incubated at 37°C for 3 h. The reactions were analyzed by HPLC using an RP 18 column (125 × 8 mm, 1 ml/min) with buffer C and a gradient of acetonitrile according to the following equation:

$$P_{AN}(t) = P_{AN1} + (P_{AN2} - P_{AN1})(e^{2t/T} - 1/e^2 - 1)$$

where $T = 10$ min, $P_{AN1} = 0\%$ CH₃CN and $P_{AN2} = 13\%$ CH₃CN

Determination of T_m values

Thermal melting curves were obtained in buffer E using a Hitachi 150-20 spectrophotometer (Japan). To determine the T_m values the first derivative was calculated.

Circular dichroism experiments

CD spectra were measured in buffer E using a Roussel-Jouan III dichrograph (France).

Hydrolysis of DNA duplexes by *EcoRII* and *SsoII*

Enzymatic cleavage was performed by incubating the ³²P-labelled substrates with 0.04 U *EcoRII* in 10 μl buffer F or 1 U *SsoII* enzyme in 10 μl G at 10°C for 60 min. The concentration of DNA duplexes (C_D) was 0.35 μM. The concentration of the activator DNA duplex IX was 0.35 μM.

Analysis of cleavage products

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

Determination of kinetic parameters

Initial velocities for cleavage of duplexes I and II by *SsoII* endonuclease were determined by spectrophotometric monitoring of the reaction (18). For that, 27 nM *SsoII* and DNA duplexes I or II in concentrations of 0.2–1.8 mM were mixed in 110 μM buffer F. The time-dependent increase in the UV absorbance at 260 nm was recorded on a Hitachi 150-20 spectrophotometer (Japan). Kinetic parameters were obtained from a Michaelis-Menten plot using non-linear regression analysis (SigmaPlot for Windows).

RESULTS AND DISCUSSION

Substrate design

Sequence-specific recognition between proteins and DNA is essentially based on contacts between functional groups on proteins and functional groups on the heterocyclic bases. 2-AP is a well-known nucleobase analogue with interesting biological properties. It can be viewed as an analogue of guanine in which the oxygen at position 6 is replaced by hydrogen. On the other hand, 2-AP is a structural isomer of adenine in which the amino group has been displaced from position 6 to position 2. In fact, 2-AP is able to form stable base pairs (mispairs) with both cytosine and thymine residues (19).

In this study we site-specifically incorporated 2-AP in place of adenine (duplex II) or guanine (duplexes III–VIII) into the recognition sites of *EcoRII* and *SsoII* restriction endonucleases (Table 1).

The oligonucleotides were synthesized by a phosphoramidite approach using a fully protected phosphoramidite building block 1 of the 2-aminopurine-2'-deoxyribose. Recently we described a very efficient new procedure for synthesis of 2-aminopurine-2'-deoxyribose by simultaneous reduction of both the 6-

Table 1. Melting temperatures and cleavage of DNA duplexes containing 2-aminopurine by restriction endonucleases *Eco*RII and *Sso*II (X = 2-AP)

Number	DNA duplex	Rel. cleavage (%) for 1h C_D 0.35 μ M			T_m , °C *
		<i>Eco</i> RII		<i>Sso</i> II	
		in the absence of canonical substrate	in the presence of duplex IX (C_D of IX 0.35 μ M)		
I	5' - GCCAACCTGGCTCT - 3'	100	-	100	62
	3' - CGGTTGGACCGAGA - 5'	100	-	100	
II	5' - GCCAACCTGGCTCT - 3'	10	60	90	56
	3' - CGGTTGGXCCGAGA - 5'	0	60	80	
III	5' - GCCAACCTXGCTCT - 3'	0	5	0	34.4, 44.0
	3' - CGGTTGGACCGAGA - 5'	0	10	0	
IV	5' - GCCAACCTGXCTCT - 3'	0	0	0	26.0; 50.0
	3' - CGGTTGGACCGAGA - 5'	0	0	0	
V	5' - GCCAACCTXXCTCT - 3'	0	0	0	23.0; 36.0
	3' - CGGTTGGACCGAGA - 5'	0	0	0	
VI	5' - GCCAACCTGGCTCT - 3'	0	0	0	34.0; 42.5
	3' - CGGTTGXACCGAGA - 5'	0	0	0	
VII	5' - GCCAACCTGGCTCT - 3'	0	0	0	37.0; 44.5
	3' - CGGTTXGACCGAGA - 5'	0	0	0	
VIII	5' - GCCAACCTGGCTCT - 3'	0	0	0	27.0; 38.0
	3' - CGGTTXXACCGAGA - 5'	0	0	0	

C_D , concentration of DNA duplexes.

*For DNA duplexes III–VIII melting temperatures at each step of biphasic curves have been determined.

and the 2'-positions of 2'-phenoxythiocarbonylated 6-chloroguanosine with tri-*n*-butyltin hydride (12).

In the first category we prepared a substrate analogue in which the central A·T base pair of the recognition sequence is replaced by a 2-AP·T base pair. Like the corresponding natural A·T pair, 2-AP·T exists as a normal Watson–Crick base pair with two hydrogen bonds. In contrast to the A·T base pair, however, the amino group of 2-AP is now pushed into the minor groove of the double-stranded DNA. This permits an investigation into the role of this amino group in the interaction of *Eco*RII and *Sso*II with the DNA.

The second category represents substrates containing 2-AP instead of two different guanine residues in the target sequence. In comparison with the natural G·C base pair the system of hydrogen bonds in the 2-AP·C base pair is changed (19–21). Consequently, it may be suggested that the structural distortion of DNA at the point of modification causes a decrease in free energy of transformation of the double-stranded DNA to the activated state during specific enzyme–substrate complex formation. In addition, this modification makes it possible to reveal the role of the inner and outer guanine residue in sequence-specific hydrolysis.

Determination of T_m values and circular dichroism measurements

Comparison of the melting behaviour of the non-modified duplex I and those of the modified duplexes II–VIII revealed the effect

of replacing G and A by 2-AP on the thermodynamic stability (Table 1).

Introduction of 2-AP instead of G in the *Eco*RII (*Sso*II) recognition site (duplexes III–VII) resulted in a biphasic melting curve (Fig. 1). This is apparently due to a distortion of the helix structure produced by the 2-AP·C base pair. The 2-AP·C base pair may adopt either a wobble structure stabilized by two hydrogen bonds or a G·C-like structure with one hydrogen bond (20). The latter is thought to be implausible. As a consequence of the significant distortion of double helix geometry in the centre of the recognition site each 14 bp DNA duplex with a 2-AP·C mismatch in the middle (III–VIII) melts as two independent halves. Extremely low T_m values of DNA duplexes III–VIII strongly suggest that melting of duplexes shorter than 14 bp occurs (Table 1). Such a two-step melting behaviour was also observed in the case of a 14 bp duplex with a nick in the middle of one of the strands (22). Very low T_m values were reported by McLaughlin *et al.* (23) for 2-AP-containing 10 bp and 12 bp duplexes and this, in our opinion, also reflects the destruction of hydrogen bonding in a 2-AP·C base pair and independent melting of the duplex fragments.

In contrast, the incorporation of 2-AP instead of A (duplex II) did not result in a biphasic melting (base pairing between 2-AP and T occurs through two hydrogen bonds, as in the case of A and T). Thus the thermodynamic stability is only slightly changed compared with the non-modified DNA duplex I (Table 1).

In order to investigate the influence of 2-AP·T or one or two 2-AP·C base pairs on the helix structure, CD spectra were

Figure 1. Melting curve of DNA duplex V in buffer E ($c = 5.0 \times 10^{-5}$ M). First derivative plots of the curve are indicated by dotted lines.

measured for duplexes I–III and V. CD spectra of these DNA duplexes (Fig. 2) resemble those of DNA and double-stranded oligonucleotides in the standard B-form. It confirms that all the DNA duplexes containing 2-AP exist largely in a B-like form.

2-AP base paired with T in a DNA helix (duplex II) has little influence on the CD spectra compared with the CD spectrum of duplex I (Fig. 2, curves 1 and 2).

Nevertheless, CD spectra of DNA duplexes III and V, in which one or two G·C base pairs are substituted by a 2-AP·C base pair, differ slightly from the spectra of duplex I (Fig. 2, curves 3 and 4). As already mentioned above, these experiments also support the assumption that the introduction of 2-AP·C causes local structural distortions of B-form DNA.

Interaction of *Eco*RII restriction endonuclease with modified DNA duplexes

The cleavage of modified substrates II–VIII by *Eco*RII endonuclease was studied (Table 1). Replacement of the central A·T base pair in the recognition site by a 2-AP·T base pair (duplex II) decreases hydrolysis of the non-modified strand and inhibits hydrolysis of the modified strand (Fig. 3, lanes 4 and 5). A similar effect is observed for *Eco*RI (GAATTC) restriction endonuclease, where substitution of 2-AP for adenine at the inner or outer base pair of the recognition site resulted in an inhibition of hydrolysis (24,25).

Recently we reported that the resistance of some synthetic DNA duplexes to *Eco*RII cleavage can be overcome by incubation with a susceptible substrate (9). This is also true for substrate II. Incubation of substrate II with *Eco*RII in the presence of substrate IX greatly enhanced cleavage of both strands (Fig. 3, lanes 6 and 7). Previously it was shown that an N^6 -methyladenine-containing substrate analogue for *Eco*RII (9) is also cleaved in the presence of the canonical substrate IX (Table 1). However, *Eco*RII does not cleave N^6 -methyladenine-containing substrate alone. This indicates that *Eco*RII interacts with the exocyclic amino group of the adenine residue in the target sequence. In terms of the proposed two-substrate mechanism of DNA hydrolysis by *Eco*RII, this enzyme interacts with four central nucleosides, including two adenine residues. However, hydrolysis is effective with one modified and other canonical sites. Hence, for effective hydrolysis *Eco*RII endonuclease requires only one amino group in the 'native' position.

The replacement of one or two guanine residues in the recognition sequence by 2-AP (DNA duplexes III–VIII) fully inhibits cleavage of these duplexes by *Eco*RII restriction

Figure 2. CD spectra of duplexes I (1), II (2), III (3) and V (4) (C_D 2.8 μ M) in 15 mM sodium citrate, pH 7.25, 150 mM NaCl.

Figure 3. Cleavage of DNA duplexes I (lanes 2 and 3) and II (lanes 4 and 5) in the presence of substrate IX (lanes 6 and 7) by *EcoRII*. Lane 1, duplex I. The ^{32}P label is in the 5'→3' (lanes 1, 2, 4 and 6) or 3'→5' (lanes 3, 5 and 7) strand of DNA duplexes. The C_D of DNA duplexes was 0.35 μM . The reaction time was 1 h. Figures on the right give the length of oligonucleotides (bp).

Duplex IX: ACCTACCTGGTGGT
TGGATGGACCACCA

Figure 4. Cleavage of DNA duplexes I (lanes 2 and 3) and II (lanes 4 and 5) by *SsoII*. Lane 1, duplex I. The ^{32}P label is in the 5'→3' (lanes 2 and 4) or 3'→5' (lanes 1, 3 and 5) strand of DNA duplexes. The C_D of DNA duplexes was 0.35 μM . The reaction time was 1 h. Figures on the right give the length of oligonucleotides (bp).

endonuclease. In the presence of the canonical substrate IX cleavage of duplexes IV–VIII is also inhibited, while in the case of duplex III weak hydrolysis by *EcoRII* was observed (Table 1). These results indicate that contacts of the *EcoRII* endonuclease with all the oxygen atoms at position 6 of the guanine residues are important for formation of the specific enzyme–substrate complex. Assuming that two recognition sites of DNA are involved in *EcoRII*–substrate interaction, eight contacts with the oxygen atoms of the guanine residues occur in the specific active complex. Only in the case of the inner guanine residue, neighbouring a central thymine of the recognition site, is the contact of *EcoRII* to the oxygen atoms of G less crucial.

Interaction of restriction endonuclease *SsoII* with modified DNA duplexes

The modified DNA duplex II containing the 2-AP·T base pair at the central position of the recognition site is cleaved by *SsoII* endonuclease (Fig. 4, lanes 4 and 5).

The kinetic results of replacing the central adenine by 2-AP are shown in Table 2. The constants k_{cat}/K_m of DNA duplexes I and II are not affected by the modification due to the coordinated decrease in both K_m and k_{cat} for the modified duplex II. The

catalytic efficiency of duplex II decreases due to a considerable decrease in k_{cat} , while the binding of *SsoII* endonuclease to the substrate analogue II does not decrease at all.

Table 2. Kinetic parameters of *SsoII* endonuclease (X = 2-AP)

Substrate ^a	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)
I ...CCTGG...			
...GGACC...	(0.327 ± 0.043)	(1.1 ± 0.4)	2.97×10^5
II ...CCTGG...			
...GGXCC...	(0.0099 ± 0.0060)	(0.33 ± 0.08)	3.00×10^5

^aRecognition sites only are indicated.

Recently it has been shown that replacement of A or T residues in the centre of the *SsoII* target sequence by a propylene bridge or 1,2-dideoxy-D-ribofuranose (non-nucleotide inserts) results in a considerable increase in the *SsoII* cleavage efficiency of each strand of the DNA duplex (11). In addition, the enzyme produced non-canonical breaks side by side with canonical ones (11). Replacement of the central A·T base pair by 2-AP·T does not result in a change of cleavage specificity of *SsoII* endonuclease. In contrast to a non-nucleotide insert, the 2-AP·T base pair in the centre of the recognition site does not disturb the conformation of the DNA. This confirms the suggestion that only local structural agility of double helix DNA produced by non-nucleotide replacements leads to non-canonical breaks and to an increase in the cleavage efficiency of *SsoII*.

Taken together, these results confirm that *SsoII* does not interact with each base of the central degenerate base pair. Probably, the enzyme recognizes only the secondary structure of the central base pair of the *SsoII* site.

Using the DNA duplexes containing 2-AP instead of one or two guanine residues in the target sequence (III–VIII) as substrates for *SsoII* endonuclease, no cleavage was observed (Table 1). Nevertheless, all substrate analogues show a competitive inhibition (data not shown), suggesting that DNA duplexes containing 2-AP·C base pairs can bind to the *SsoII* enzyme. This result differs from our earlier findings that DNA duplexes containing a non-nucleotide insert (1,2-dideoxy-D-ribofuranose) instead of one of the guanine residues in the recognition sequence are cleaved by *SsoII* with increased efficiency, producing non-canonical breaks in the modified strand (11).

In contrast to 1,2-dideoxy-D-ribofuranose, the replacement of G by a 2-AP residue preserves all functional groups except oxygen at position 6 of the guanine residue. However, in addition to Watson–Crick base pairing with one hydrogen bond, 2-AP opposite C is capable of forming a 'wobble' pair in which two hydrogen bonds exist (21). This would lead to a drastic local distortion of DNA conformation at the point of modification. It seems highly plausible that the enzyme does not recognize this 'wobble' pair at all. To this end, we suggest that both the inner and outer G residues within the recognition site are very important for the formation of the transient 'open' state of DNA within the enzyme–substrate complex and for the specific hydrolysis by *SsoII* endonuclease.

CONCLUSIONS

The interaction of restriction endonucleases *EcoRII* and *SsoII* with two categories of substrate analogues, 2-AP·T- and

2-AP·C-containing DNA duplexes, has been studied. Restriction endonuclease *SsoII* recognizes and cleaves the modified duplex containing 2-AP in place of an adenine residue in the central position of the recognition site. Furthermore, this modified substrate is cleaved efficiently by *EcoRII* in the presence of the canonical substrate. In contrast, 2-AP·C in the recognition site inhibits the hydrolysis of modified DNA duplexes by *EcoRII* and *SsoII* endonucleases. We assume that this replacement leads to a change in DNA conformation followed by distortion of the specific contacts between *EcoRII* enzyme and DNA, which prevents formation of the active complex.

Earlier it was shown (11) that non-nucleotide inserts in place of G in the recognition site resulted in an increase in efficiency of *SsoII* hydrolysis. It has been suggested that introduction of a non-nucleotide insert into the recognition site may affect the formation of the DNA transition state ('open' form) which takes place in the *SsoII*-substrate complex. Besides, DNA duplexes containing a non-nucleotide insert in place of adenine, as well as in place of guanine, in the recognition site are effectively cleaved by *EcoRII* enzyme in the presence of the canonical substrate (25). Thus the structural dynamics of DNA duplexes with an 'abasic site' allows this substrate analogue to be included in the catalytic enzyme-substrate complex. In contrast, a 'wobble' 2-AP·C base pair in the recognition site prevents appropriate conformational rearrangements of the substrate.

These results are confirmed by CD data and thermal denaturation experiments. No significant differences in the melting behaviour and CD spectra between DNA duplex I and one with a 2-AP·T replacement were observed. Nevertheless, structural destabilization, biphasic melting curves and a slightly modified CD spectra have been observed in the case of 2-AP·C replacement.

In this work the important role of guanine residues and the structure of the G·C base pair within the recognition site have been demonstrated for specific interaction of *EcoRII* and *SsoII* enzymes with DNA.

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