Cross-linking of *Sso*II restriction endonuclease to cognate and non-cognate DNAs

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Abstract Specific and non-specific interactions of *Sso*II restriction endonuclease (R·*Sso*II) were probed by the method of covalent attachment to modified DNA containing an active monosubstituted pyrophosphate internucleotide bond instead of a phosphodiester one. R·*Sso*II with six N-terminal His residues was shown to be cross-linked to duplexes with this type of modification, either containing or not the recognition sequence. Competition experiments with covalent attachment of R·*Sso*II to activated DNAs demonstrated the similar affinity of the enzyme to cognate and non-cognate DNAs in the absence of cofactor, Mg^{2+} ions.

Key words: DNA-protein interaction; Restriction endonuclease; Duplex with active internucleotide group; Covalent attachment

1. Introduction

Recently it was established that a number of restriction endonucleases require Mg^{2+} ions, not only for catalysis but also for the specificity of substrate recognition [1]. For example, restriction endonuclease *Eco*RV interacts with cognate and non-cognate DNAs with nearly equal affinity in the absence of cofactor [2], as revealed by nitrocellulose binding or electrophoretic mobility shift assay. In the present paper we investigated the specific and non-specific interactions of the restriction endonuclease *Sso*II (R·*Sso*II) with DNAs by the method of covalent attachment of the enzyme to activated DNA. We chose DNA duplexes containing a monosubstituted pyrophosphate internucleotide bond instead of a phosphodiester one in the enzyme recognition site as reagents for affinity modification of R·*Sso*II. These activated DNA duplexes were recently shown to successfully modify the active centers of restriction endonucleases *Rsr*I, *Eco*RI [3], *Eco*RII [4,5] and *Mva*I [5]. Covalent attachment may occur on the binding or catalytic step depending on cross-linking conditions.

2. Materials and methods

2.1. Enzymes and oligonucleotides

R·*Sso*II (10000 U/ml) was purified from *E. coli* M15 cells carrying the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible overexpression plasmid pQE*Sso*9 [6]. This plasmid contained the R·*Sso*II gene [7] in the pQE9 tac expression vector (DIAGEN GmbH, Germany). The *Sso*II endonuclease encoded by pQE*Sso*9 plasmid possessed an N-terminal 6×His affinity tail, making it possible to purify the enzyme to virtual homogeneity by one-step Ni-chelate affinity column chromatography. The introduced amino acid substitutions do not change R·*Sso*II recognition and cleavage properties if compared with the unmodified enzyme obtained earlier [8].

Oligonucleotide precursors were synthesized by T.S. Oretskaya, E.M. Volkov and E.A. Romanova as described in [9]. DNA duplexes with a monosubstituted pyrophosphate internucleotide bond for cross-linking to R·*Sso*II were synthesized as described in [10]. DNA duplex I with an ethoxy or isopropoxy group and DNA duplexes II and III with an ethoxy group in modified internucleotide linkage were obtained (Table 1). DNA duplexes were ^{32}P-labeled by T4 polynucleotide kinase. ^{32}P label was at the monosubstituted phosphate group of the modified internucleotide linkage of the DNA duplex modified strand (see formulae) and at the 5′ end of the non-modified strand. Cleavage analysis of DNA duplexes by R·*Sso*II was performed as described in [11].

2.2. Cross-linking experiment

Cross-linking of R·*Sso*II (concentration per monomer 1.4×10^{-6} M) to substrates I–III (concentration per duplex 1.8×10^{-7} M) was performed in 20 μl of buffer A: 10 mM *N*-methylimidazole (MeIm), pH 7.5, 50 mM NaCl, 0.1 mM dithiothreitol (DTT) and 0, 0.15 or 15 mM $MgCl_2$, or B: 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM DTT and 0, 0.15 or 15 mM $MgCl_2$ at 37°C for 18 h. DNA duplexes I–III contained ^{32}P-label only at the monosubstituted phosphate group of modified internucleotide linkage (see formulae). Reactions were followed by 0.1% SDS–10% PAGE [12] after heating samples in 0.1% SDS–2-mercaptoethanol solution at 95°C. The gels were analyzed by autoradiography and staining with Coomassie blue. The coincidence of the radioactive band and the band containing protein proved the formation of the DNA-enzyme covalent complex. Cross-linking yield was determined as the ratio of the radioactivity of the DNA-enzyme covalent complex to the total radioactivity of the conjugate and the unbound DNA. The average results for cross-linking of R·*Sso*II to duplexes I and II are reported in Table 2.

2.3. Competition inhibition of R·SsoII cross-linking to duplexes with a monosubstituted pyrophosphate internucleotide bond

Competition inhibition of R·*Sso*II cross-linking to duplexes I and II was studied in the presence of increasing amounts of unlabeled DNA duplexes IV and V added to reach ratios of molar concentrations of inhibitor and reagent equal to 0.5, 1, 2, 3, 4, 5, 7, 10, 14, 21, 28. Conditions of the reactions: buffer B, 37°C, 18 h.

3. Results and discussion

R·*Sso*II recognizes the double stranded sequence

5′ ...↓ CCNGG ... 3′
3′ ... GGNCC↑ ... 5′

degenerated at the central position and cleaves it as shown by arrows.

For the affinity modification of R·*Sso*II, we used ^{32}P-labeled DNA duplexes I and II containing the recognition site of this

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Abbreviations: MeIm, *N*-methylimidazole; DTT, dithiothreitol

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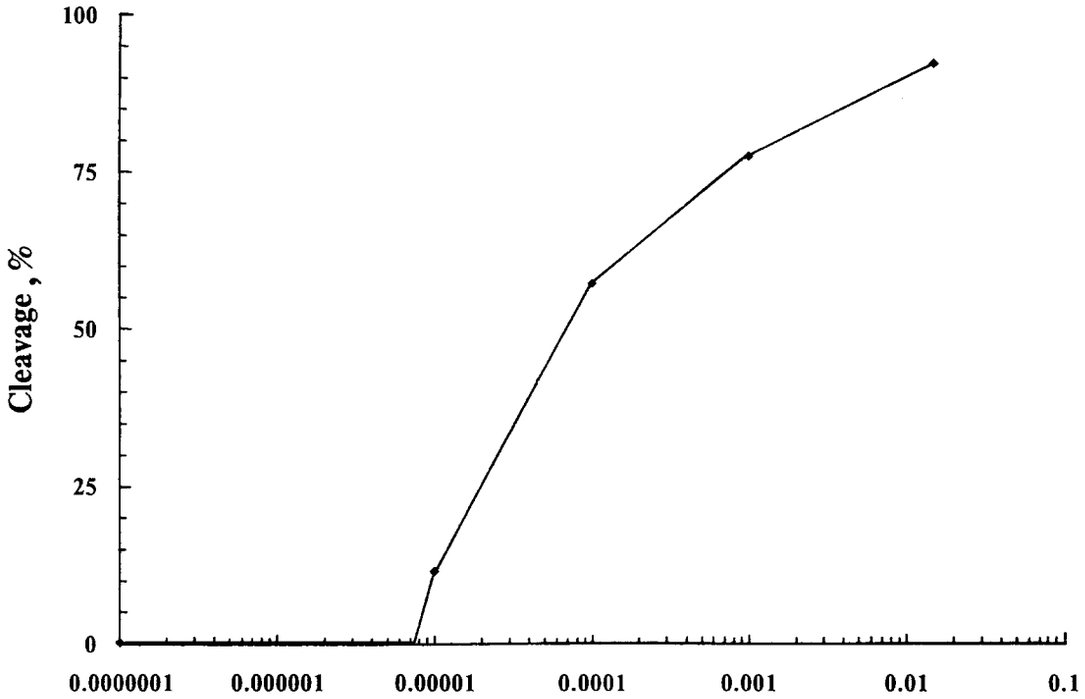
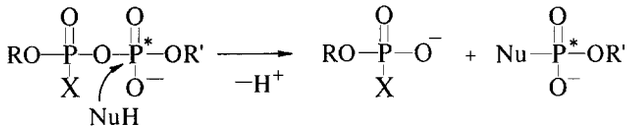


Fig. 1. Dependence of 14-mer canonical DNA duplex cleavage extent by R·*Sso*II on Mg^{2+} ion concentration. Cofactor concentration is adduced in logarithmic scale. 37°C, 1 h, buffer B.

enzyme (Table 1), ^{32}P label being located in the disubstituted phosphate group of the pyrophosphate. DNA duplex I contained the activated pyrophosphate group in the middle of the R·*Sso*II recognition sequence (marked in bold). In duplex II, the monosubstituted pyrophosphate bond was adjacent to the recognition site and coincided with the R·*Sso*II scissile bond. Covalent attachment of the nucleophilic agent to the modified strand of the duplex follows the scheme



where NuH is a nucleophilic agent, RO and R'O are oligonucleotide residues, X is a non-nucleotide substituent, and P* is the ^{32}P label.

Ethoxy or isopropoxy groups were chosen as non-nucleotide substituents. The introduction of an additional methyl group into the non-nucleotide substituent of the modified internucleotide linkage does not influence the interaction between activated DNA and proteins (unpublished results). The nucleophilic amino acid side chains of R·*Sso*II are supposed to be attached to oligonucleotide *pTGGTGGT from duplex I or *pCCTGGATCCG from duplex II during the cross-linking reaction [10].

The capability of R·*Sso*II to bind DNAs with modification of the sugar-phosphate backbone was demonstrated by the gel retardation assay (data not shown). Introduction of a monosubstituted pyrophosphate bond does not prevent the hydrolysis of duplexes I and II by R·*Sso*II (Table 1).

The data on cross-linking of oligonucleotides from duplexes I and II to R·*Sso*II are adduced in Table 2. One can see that the yield of cross-linking varies from 2 to 22% in buffer B (Tris-HCl buffer) in the absence of cofactor, Mg^{2+} ions. We tried to get higher yields of cross-linking with the help of the nucleophilic catalyst MeIm which was previously shown to increase the efficiency of restriction endonuclease attachment to the same type of activated DNA [3–5]. But the presence of MeIm did not result in an increase of the extent of cross-linking of R·*Sso*II to duplexes I and II in comparison with

Table 1
R·*Sso*II hydrolysis of DNA duplexes I and II in buffer B

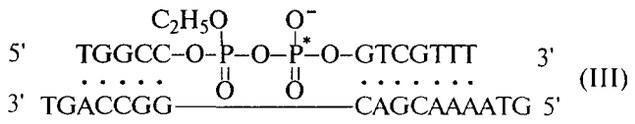
N	DNA duplex	Strand	Cleavage, %
I*)	5' ACCTACC–O–P(AlkO)–O–P*(O⁻)–O–TGGTGGT 3' 3' TGGATGG————ACCACCA 5	a b	29 34
II	5' GTCACT–O–P(C_2H_5O)–O–P*(O⁻)–O–CCTGGATCCG 3' 3' CAGTGA————GGACCTAGGCA 5	a b	**) 51

*Alk=iC_3H_7.
**The extent of hydrolysis was not determined due to the coincidence of the scissile bond and the modified internucleotide bond.

that in Tris-HCl buffer (Table 2).

There is a tendency that introduction of Mg^{2+} ions causes a decrease of cross-linking of R·*Sso*II to duplex I. Comparing data of Table 2 and Fig. 1, one can conclude that this decrease is connected with substrate hydrolysis at Mg^{2+} concentrations higher than 1.5×10^{-4} M. In the case where the modified internucleotide bond coincides with the scissile bond (duplex II), addition of Mg^{2+} ions does not influence the cross-linking yield.

To investigate the specificity of R·*Sso*II cross-linking to duplexes I and II we (i) checked the possibility of affinity modification of R·*Sso*II by activated DNA duplex III without the R·*Sso*II recognition site



(III)

and (ii) tested the possibility of inhibition of cross-linking of reagents (DNA duplexes I and II) by non-modified duplexes with (IV) or without (V) recognition site

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5'  GCCAACCTGGCTCT  3'        (IV)
3'  CGGTTGGACCGAGA  5'

5'  GCTGGTGGCTCGAC  3'
3'  CGACCACCGAGCTG  5'        (V)
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Duplex IV had a different nucleotide sequence from that of duplexes I and II in order to prevent overcomplicated effects of strand exchange during the cross-linking reaction in the presence of both reagent and inhibitor.

The ability of duplex III to modify R·*Sso*II was shown with a cross-linking yield of 11% (buffer B without Mg^{2+} ions). The results of the competition inhibition by duplexes IV and V in buffer B without Mg^{2+} ions are shown in Fig. 2. A 7-fold excess of substrate IV completely inhibits the covalent attachment of R·*Sso*II to duplex I. However, the trend to a decrease of the yield of the DNA-protein covalent complex is observed also in the presence of duplex V.

We consider the covalent attachment of substrate to enzyme to be specific when it occurs within the catalytic domain of the protein. The inhibition of the cross-linking reaction of R·*Sso*II

Table 2
Dependence of the extent of R·*Sso*II affinity modification by duplexes I and II on buffer solution content, 37°C, 18 h (%)

Duplex	Yield of cross-linking (%)					
	Conditions					
	MeIm buffer*			Tris-HCl buffer**		
	$MgCl_2$ concentration (M)			$MgCl_2$ concentrantion (M)		
	0	1.5×10^{-4}	1.5×10^{-2}	0	1.5×10^{-4}	1.5×10^{-2}
I***	19	17	2	22	20	5
II	5	–	5	2	–	6

*Buffer A, see Section 2.2.
**Buffer B, see Section 2.2.
***Alk=C_2H_5.

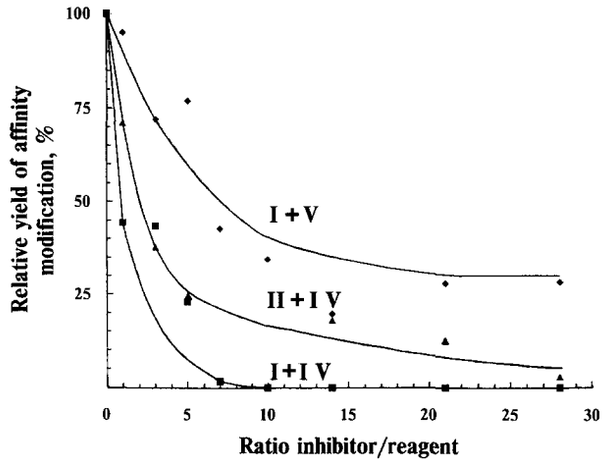

Fig. 2. Dependence of affinity modification of R·*Sso*II by duplexes I (Alk = iC_3H_7) and II on the ratio of duplexes IV or V to I or II. Concentration of duplexes I and II per duplex is 1.8×10^{-7} M. Yield of R·*Sso*II affinity modification by duplexes I and II in the absence of IV and V is referred to as 100%. 37°C, 18 h, buffer B.

to duplexes I and II by substrate IV demonstrates that the covalent bond between the enzyme and DNA is formed at the active site of the enzyme.

DNA duplex V also inhibits the covalent attachment of R·*Sso*II to duplex I though at a higher concentration (Fig. 2). The inhibition of cross-linking by duplex V as well as the successful attachment to duplex III demonstrates that non-cognate DNAs can also interact with the active center of the protein. We suggest that interaction of R·*Sso*II with cognate and non-cognate DNAs appears in the same protein domain. R·*Sso*II has the higher affinity to substrates which can be accounted as K^S/K^I to be equal to $[I]_{50}/[R]_{50}/[S]_{50}/[R]_{50}$, where K^S and K^I are constants of specific and non-specific association, $[I]_{50}/[R]_{50}$ and $[S]_{50}/[R]_{50}$ are ratio inhibitor/reagent for non-cognate (V) and cognate (IV) inhibitor respectively, at which cross-linking efficiency decreases 2-fold. This value is equal approximately 3/1 (see Appendix A).

It is known that all DNA acting enzymes recognize non-specific DNA sequences. However, usually the constant of specific interaction differs from the non-specific one by 2 [13,14] or even 4–5 [15] orders of magnitude as was established by kinetic investigations [13] or binding assays on nitrocellulose filters [14,15]. From this point of view, it is interesting to note that R·*Sso*II constants of specific and non-specific interaction with DNA have the same order of magnitude in the absence of Mg^{2+} ions.

So by the method of cross-linking of R·*Sso*II with the six N-terminal His residues to duplexes with a monosubstituted pyrophosphate internucleotide bond, it was established that the enzyme is a member of the family of restriction endonucleases that require Mg^{2+} not only for the catalytic act, but also for specific recognition of cognate DNA.

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Appendix

Calculation of the ratio of constants of specific and non-specific association

Consider the case when two equilibria occur

$$ES \leftrightarrow {}^{S}E \leftrightarrow {}^{R}ER \rightarrow ER_{CL}$$

where E is enzyme; S is substrate; R is reagent for cross-linking; ER_{CL} is product of covalent attachment of the enzyme to reagent; ER and ES are enzyme-reagent and enzyme-substrate complexes respectively.

Thermodynamic constants can be written as

$$K^R = \frac{[ER]}{[E][R]}; \quad K^S = \frac{[ES]}{[E][S]}$$

for enzyme-reagent and enzyme-substrate complexes respectively.

Their ratio will be equal to

$$\frac{K^R}{K^S} = \frac{[ER][E][S]}{[ES][E][R]} = \frac{[ER][S]}{[ES][R]}$$

A 2-fold decrease of cross-linking indicates that half of the busy active centers are occupied by the substrate rather than reagent, in this case [ES]=[ER] and

$$\frac{K^R}{K^S} = \frac{[S]_{50}}{[R]_{50}}$$

where $[S]_{50}/[R]_{50}$ is ratio substrate/reagent (i.e. duplex IV/duplex I) in the case where cross-linking efficiency is equal to 50% of the initial (or decreases 2-fold).

In the similar case

$$EI \leftrightarrow {}^{I}E \leftrightarrow {}^{R}ER \rightarrow ER_{CL}$$

where I is non-cognate DNA inhibitor and EI is complex of the enzyme with non-cognate DNA inhibitor (i.e. duplex V),

$$\frac{K^R}{K^I} = \frac{[I]_{50}}{[R]_{50}}$$

where $[I]_{50}/[R]_{50}$ is ratio inhibitor/reagent (i.e. duplex V/duplex I) in the case where cross-linking efficiency is equal to 50% of the initial (or decreases 2-fold). Distributing K^R/K^I by K^R/K^S we get

$$\frac{K^R/K^I}{K^R/K^S} = \frac{K^S}{K^I} = \frac{[I]_{50}/[R]_{50}}{[S]_{50}/[R]_{50}}$$

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