*Eco*RII endonuclease has two identical DNA-binding sites and cleaves one of two co-ordinated recognition sites in one catalytic event

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Abstract ***Eco*RII is a typical restriction enzyme that cleaves DNA using a two-site mechanism. *Eco*RII endonuclease is unable to cleave DNA which contains a small number of *Eco*RII recognition sites but the enzyme activity can be stimulated in the presence of DNA with a high frequency of *Eco*RII sites. To investigate the mechanism of activation, the kinetics of stimulated *Eco*RII cleavage has been studied. A 14 bp substrate activated the cleavage of the 71 bp substrate, containing one *Eco*RII recognition site (*trans*-activation) by a competitive mechanism: the activator increased substrate binding but not catalysis. The activation increased if the substrate concentration decreased and if the activator had a lower affinity for the enzyme than the substrate. The introduction of the second recognition site into the 71 bp duplex also enabled cleavage of this substrate (*cis*-activation). Pyrophosphate bonds were incorporated into one of two recognition sites to switch off the cleavage of the phosphodiester bonds. Analysis of cleavage products of these modified substrates showed that *Eco*RII cuts one of two coordinated recognition sites in one catalytic event.**

Key words: *Eco*RII endonuclease; Activation mechanism; Non-hydrolyzable bond

1. Introduction

Recently it was shown that some of the type II restriction endonucleases require two DNA recognition sites for catalytic complex formation [1,2]. Restriction endonuclease *Eco*RII (R·*Eco*RII) was the first example of such enzymes. It recognizes the sequence 5′-↓CCA/TGG-3′ in DNA, and cuts it as shown by the arrow. R·*Eco*RII cleaves extended DNA rather poorly if a substrate molecule contains one recognition site or sites are placed far apart [3]. However, such a cleavage is observed in the presence of short substrates, susceptible DNAs or some modified DNAs [4–7]. The enzyme-substrate active complex includes two subunits of *Eco*RII endonuclease interacting with two DNA recognition sequences [8]. The *Eco*RII-catalyzed reaction showed positive substrate cooperativity [9]. A kinetic scheme of the cooperative interaction of the enzyme with two DNA recognition sites was proposed [10].

The purposes of this study were: (i) to answer the question whether these two binding sites of the enzyme are identical in the catalytic complex or not; to investigate kinetic parameters of the reaction which define *Eco*RII activation, and (ii) to determine the number of phosphodiester bonds which the enzyme cleaves rapidly in one catalytic event.

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2. Materials and methods

2.1. DNA duplexes

Oligonucleotides were synthesized by the phosphoramidite method with 2-cyanoethyl as the phosphate protecting group. An automated DNA synthesizer (Gene Assembler Plus, Pharmacia) was used for chain assembly at a 0.2 mmol scale. Modified oligonucleotides containing a pyrophosphate bond were synthesized by chemical ligation of 5′- or 3′-phosphorylated oligonucleotides (28p-mer and p43-mer or p33-mer and 38p-mer, see sequences of 71.2pp1, 71.2pp2 and 71.2pp3) on the 47 bp template with water-soluble carbodiimide [11]. ^{32}P labeling was carried out using T4 polynucleotide kinase and [γ-^{32}P]ATP.

2.2. Enzyme

R·*Eco*RII (2 U/μl, 0.32 pmol/μl), containing six histidine residues at the N-terminus, was isolated from an overproducing strain and purified to homogeneity by N.V. Chichkova, A.G. Evstafieva, and A.B. Vartapetian.

Cleavage was performed by incubating the ^{32}P-labeled substrates with 2 U of the enzyme in 20 μl of 10 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl, 5 mM $MgCl_2$, 7 mM dithiothreitol, at 37°C for 5–30 min. Substrate and activator concentrations are indicated in the figure legends. Enzymatic reactions were stopped by adding 2 μl of 200 mM EDTA solution to a final concentration of 10 mM. The reactions were extracted with phenol/chloroform and cleavage products of ^{32}P-labeled duplexes were analyzed on 12% polyacrylamide gel containing 8 M urea. Radioactivity of gel slices was determined by Cherenkov counting. Cleavage percent was defined as a quotient of product radioactivity to total radioactivity of the product plus that of the uncleaved substrate. Velocities of cleavage were determined in the linear region for cleavage with respect to time (5 min) and effective constants (V_{max} and K_m^{eff}) were obtained by least squares analysis. Curve fitting computations were performed using regression analysis (SigmaPlot for Windows) and individual kinetic parameters (Fig. 5) were calculated according to Eqs. 2, 4 and 5 by analyzing the dependences of parameters *a*, 1/*c* and *b*/*c* on the different kinetic parameters.

3. Results

3.1. Mathematical modeling

The interaction of two *Eco*RII endonuclease subunits (E_2) with two recognition sites was described by a kinetic scheme presented in Fig. 1 [10]. One of the substrates (I) was considered an activator of the hydrolysis of substrate S. On the basis of the kinetic scheme a kinetic equation (Eq. 1) for the initial rates was deduced. An activation effect was determined as a ratio of initial rate of the substrate S hydrolysis in the presence of activator (v) to initial rate of the substrate S hydrolysis without activator (v_o).

$$\frac{v}{v_o} = \frac{1 + aI_o}{1 + bI_o + c[I_o]^2} \quad (1)$$

where:

$$a = \frac{k_{si}/K_{si}}{k_{ss}/K_{ss}} \times \frac{1}{S_o} \quad (2)$$

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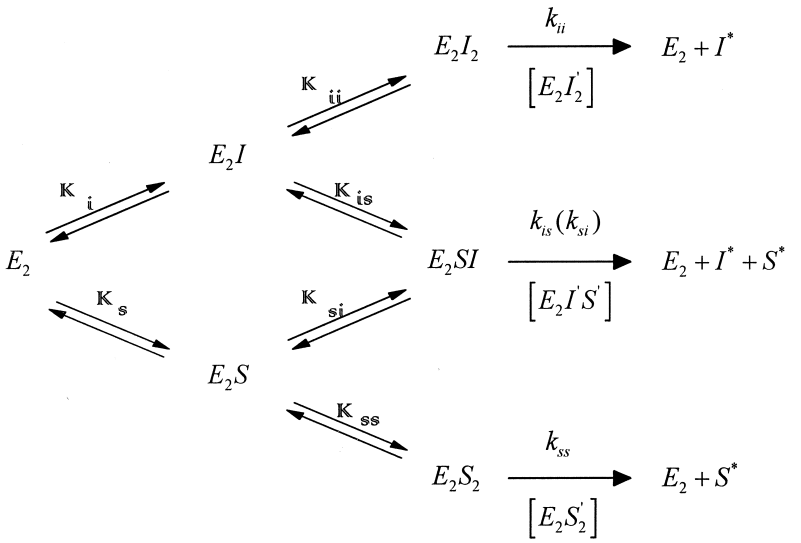


E_2 - R•*Eco*RII dimer,
E_2I, E_2I_2, E_2S, E_2S_2, E_2SI - enzyme-substrate complexes,
S, I - substrates, S^*, I^* - products of the reaction,
S', I' - transition states of the substrate,
K_i, K_s, K_{si}, K_{ii}, K_{ss} - equilibrium constants,
k_{ii}, k_{is} ($=k_{si}$), k_{ss} - rate constants.

Fig. 1. Kinetic scheme for the interaction of R·*Eco*RII with two substrates.

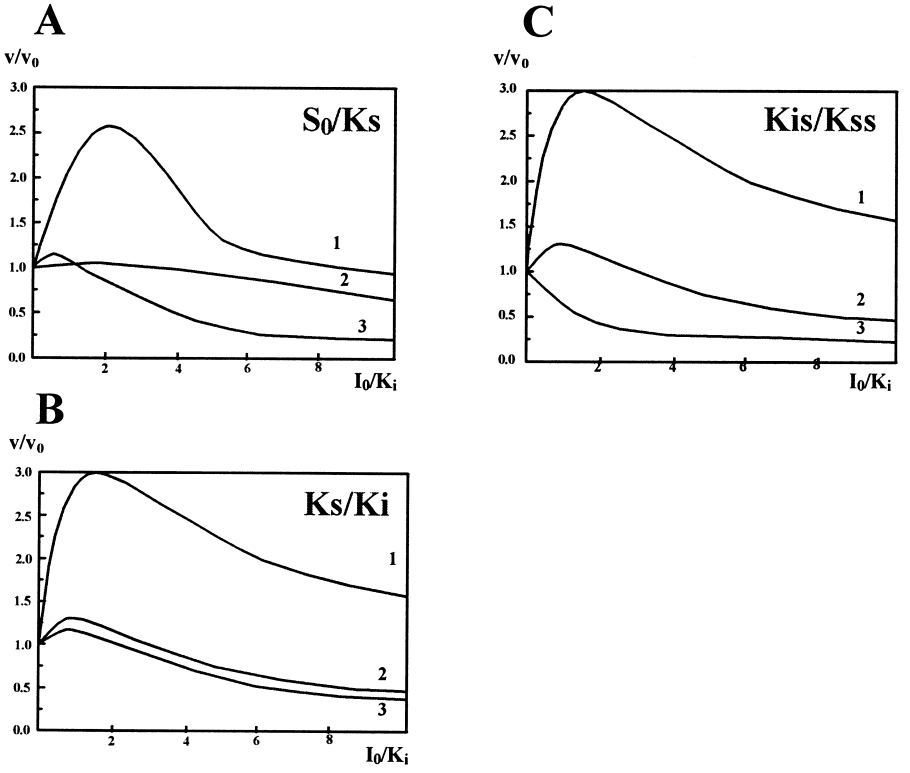


Fig. 2. Mathematical modeling of the activation effect. Varied kinetic parameters: A = S_o/K_s; B = K_s/K_i; C = K_{is}/K_{ss}. Curves 1–3 correspond to parameter values 0.1, 1.0 and 10 respectively.

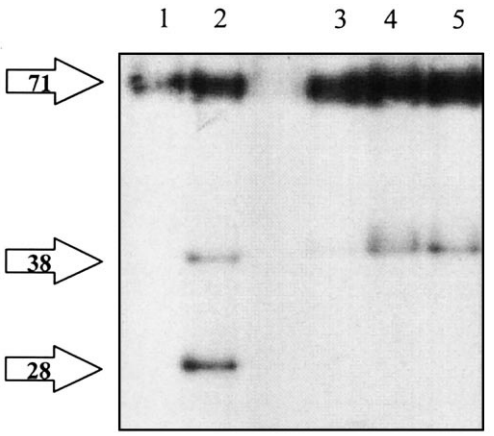


Fig. 3. Cleavage of 71 bp DNA duplexes with one or two recognition sites by R·*Eco*RII. Lane 1: duplex 71.2 without the enzyme; lanes 2 and 3: cleavage of 71.2 (25 nM) and 71.1 (50 nM) respectively; lanes 4 and 5: cleavage of 71.1 in the presence of activator 14.1 (100 and 400 nM respectively). ^{32}P is the lower strand of DNA duplexes. Reaction time, 30 min. Figures on the left indicate the length of oligonucleotides.

$$b = \frac{1}{K_i} \times \frac{1 + \frac{S_o}{K_{is}}}{1 + \frac{S_o}{K_s} + \frac{S_o^2}{K_s K_{ss}}} \quad (3)$$

$$c = \frac{1}{K_i K_{ii}} \times \frac{1}{1 + \frac{S_o}{K_s} + \frac{S_o^2}{K_s K_{ss}}} \quad (4)$$

$$b/c = K_{ii}\left(1 + \frac{S_o}{K_{is}}\right) \quad (5)$$

S_o, I_o are initial concentrations of substrate and activator.

To estimate the influence of different kinetic parameters of the *Eco*RII-activated cleavage reaction on the activation effect we examined the correlation between the activation effect (v/v_o) and the relative activator concentration (I_o/K_i) within an interval $0 \leq I_o/K_i \leq 10$ for three values for each of the parameters S_o, K_s/K_i or K_{is}/K_{ss} (0.1, 1.0 and 10) by mathematical modeling (Fig. 2). The curves had a characteristic bell-shaped form due to superposition of two effects: activation and inhibition. The activation effect of the substrate S hydrolysis was increased at decreasing substrate S concentration (Fig. 2A), increasing E_2 affinity for S (in comparison with affinity for I) (Fig. 2B) and increasing E_2I affinity for S (in comparison with E_2S affinity for S) (Fig. 2C).

3.2. Kinetics of activated cleavage of 71 bp DNA duplex by EcoRII endonuclease

To determine whether two binding sites of the *Eco*RII enzyme are identical in the catalytic complex and to investigate the activation mechanism we have constructed one-site 14 and 71 bp duplexes: 14.1 (activator) and 71.1 (substrate).

5′ ATGACCATGATTACGCCAATCAGCAGCT**CCAGG**TCGTACCTCCAGCTACCAATCCCCGGGTACCGATCTCG
3′ TACTGGTACTAATGCGGTTAGTCGTCGA**GGTCC**AGCATGGAGGTCGATGGTTAGGGGCCCATGGCTAGAGC
(71.1)

5′ ATGACCATGATTACGCCAATCAGCAGCT**CCAGG**TCGTA**CCTGG**AGCTACCAATCCCCGGGTACCGATCTCG
3′ TACTGGTACTAATGCGGTTAGTCGTCGA**GGTCC**AGCAT**GGACC**TCGATGGTTAGGGGCCCATGGCTAGAGC
(71.2)

5′ ATGACCATGATTACGCCAATCAGCAGCT**ppCCAGG**TCGTA**CCTGG**AGCTACCAATCCCCGGGTACCGATCTCG
3′ TACTGGTACTAATGCGGTTAGTCGTCGA--**GGTCC**AGCAT**GGACC**TCGATGGTTAGGGGCCCATGGCTAGAGC
(71.2pp1)

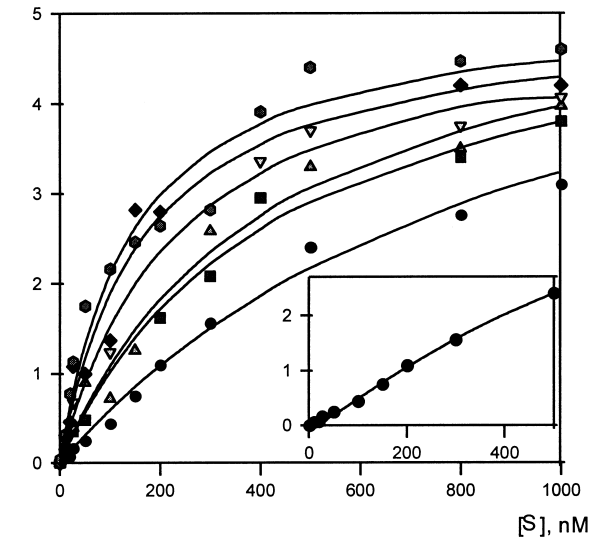
5′ ATGACCATGATTACGCCAATCAGCAGCT**CCAGG**--TCGTA**CCTGG**AGCTACCAATCCCCGGGTACCGATCTCG
3′ TACTGGTACTAATGCGGTTAGTCGTCGA**GGTCCpp**AGCAT**GGACC**TCGATGGTTAGGGGCCCATGGCTAGAGC
(71.2pp2)

5′ATGACCATGATTACGCCAATCAGCAGCT**ppCCAGG**-TCGTA**CCTGG**AGCTACCAATCCCCGGGTACCGATCTCG
3′TACTGGTACTAATGCGGTTAGTCGTCGA-**GGTCCpp**AGCAT**GGACC**TCGATGGTTAGGGGCCCATGGCTAGAGC
(71.2pp3)

5′ AGAG**CCAGG**TTGGC
3′ TCTC**GGTCC**AACCG
(14.1)

Bold face - *Eco*RII recognition sites, pp - pyrophosphate bond

A



B

N	Inductor concentration, nM	V_{max}, nM/min	K_m^{eff}, nM
1	0	5.2±0.6	740±140
2	20	5.7±0.7	430±100
3	80	5.6±0.8	410±120
4	150	5.0±0.4	230±50
5	300	4.8±0.8	170±70
6	500	5.1±0.4	140±30

Fig. 4. Kinetics of activated *Eco*RII cleavage. A: Cleavage of substrate 71.1 (S) at different concentrations of activator 14.1: (circles) 0, (squares) 20, (upward triangles) 80, (downward triangles) 150, (diamonds) 300 and (hexagrams) 500 nM. B: K_m^{eff} and V_m values of substrate 71.1 hydrolysis.

We have studied the cleavage of substrate 71.1 in the presence of different amounts of activator 14.1 (Fig. 3). Duplex 71.1 was very poorly cut by R·*Eco*RII (Fig. 3, lane 3). The activator 14.1 enhanced the efficiency of cleavage (Fig. 3, lanes 4 and 5). The hydrolysis of substrate 71.1 by R·*Eco*RII was studied in multiple turnover experiments at various substrate concentrations, while maintaining fixed concentrations of activator 14.1 (Fig. 4). The dependence of duplex 71.1 cleavage on the substrate concentration in the absence of activator was sigmoidal: positive substrate cooperativity was observed at substrate S concentrations below 200 nM (Fig. 4A, curve 1). This is consistent with the model proposed by Gabbara and Bhagwat [9] in which the cleavage reaction of a short DNA duplex containing a single site by *Eco*RII enzyme showed positive substrate cooperativity. Increasing the concentration of activator 14.1 up to 500 nM also resulted in enhancement of the initial rates of substrate 71.1 hydrolysis. For the activated *Eco*RII cleavage the initial rates increased with increasing concentrations of 71.1 in hyperbolic fashion and were fitted to the Michaelis-Menten equation to yield values for V_{max} and K_m (Fig. 4, curves 2–6). However, obtained values for K_m were effective due to a two-substrate binding mechanism. The value of V_{max} was independent of activator concentration (Fig. 4B). From the average V_{max} value (about 5 nM/min) and the concentration of the R·*Eco*RII dimer (about 32 nM) the value of k_{cat} was determined to be 0.0029 s^{-1}. K_m^{eff}, however, varied with activator concentration: it decreased from 740 to 140 nM when activator concentration increased from 0 to 500 nM (Fig. 4B). The data suggest [12] that the activator affects substrate binding and the activation obeyed a competitive mode.

Dependences of the activation effect v/v_o on the activator 14.1 concentration within an interval 0 nM $\leq I_o \leq$ 3500 nM have been analyzed, while maintaining fixed substrate 71.1 concentrations (Fig. 5) in order to determine dissociation constants for inactive (E_2I and E_2S) and active (E_2S_2, E_2SI, E_2IS and E_2I_2) complexes (Fig. 1) and to estimate the kinase prerequisite of activation. According to the suggested kinetic scheme the experimental data (Fig. 5A) were approximated by Eq. 1 and parameters a, c and b/c were calculated using regression analysis. Individual kinetic parameters for the substrate 71.1 and activator 14.1 at each step of their interaction with the *Eco*RII enzyme (Fig. 5B) were calculated according to Eqs. 2, 4 and 5 by analyzing the dependences of parameters a, $1/c$ and b/c on the different kinetic parameters. K_{is} and K_{ii} were obtained from a linear plot b/c on S_o (according to Eq. 5: K_{ii} is γ intercept, K_{is} is 1/slope). K_{ss} was fixed as K_m^{eff} on the basis of

$$v_o = \frac{k_{ss}E_oS_o^2}{K_sK_{ss} + K_{ss}S_o + S_o^2}$$

(where v_o was deduced from the scheme in Fig. 1, when I_o=0) and our suggestion that $K_s \ll K_{ss}$. According to Eq. 4 a linear plot $1/c$ on $S_o(S_o+K_{ss})$ was obtained: K_iK_{ii} was determined as γ intercept and K_i was calculated; K_sK_{ss} was determined as x intercept and K_s was calculated. K_{si} was calculated from $K_iK_{is} = K_sK_{si}$ (according to the kinetic scheme, Fig. 1). According to Eq. 2 parameter

$$\frac{k_{si}/K_{si}}{k_{ss}/K_{ss}}$$

was obtained as a slope of linear plot a on $1/S_o$ and k_{si}/k_{ss} was calculated.

3.3. Cleavage of two-site 71 bp DNA duplex and its analogues containing non-hydrolyzable pyrophosphate bonds

To determine the number of phosphodiester bonds which the enzyme cleaves rapidly in one catalytic event we studied *Eco*RII cleavage of substrate 71.2 containing two recognition sites and its analogues containing non-hydrolyzable pyrophosphate (pp) bonds (71.2pp1, 71.2pp2 and 71.2pp3) in multiple turnover experiments. The introduction of the second *Eco*RII recognition site into the 71 bp sequence enabled cleavage of this two-site substrate by a *cis*-activation mechanism. Fig. 3 shows efficient cleavage of substrate 71.2 (lane 2) and very poor cleavage of substrate 71.1 (lane 3) by R·*Eco*RII, the concentration of *Eco*RII recognition sites being the same in both cases. Two cleavage products were observed after *Eco*R-

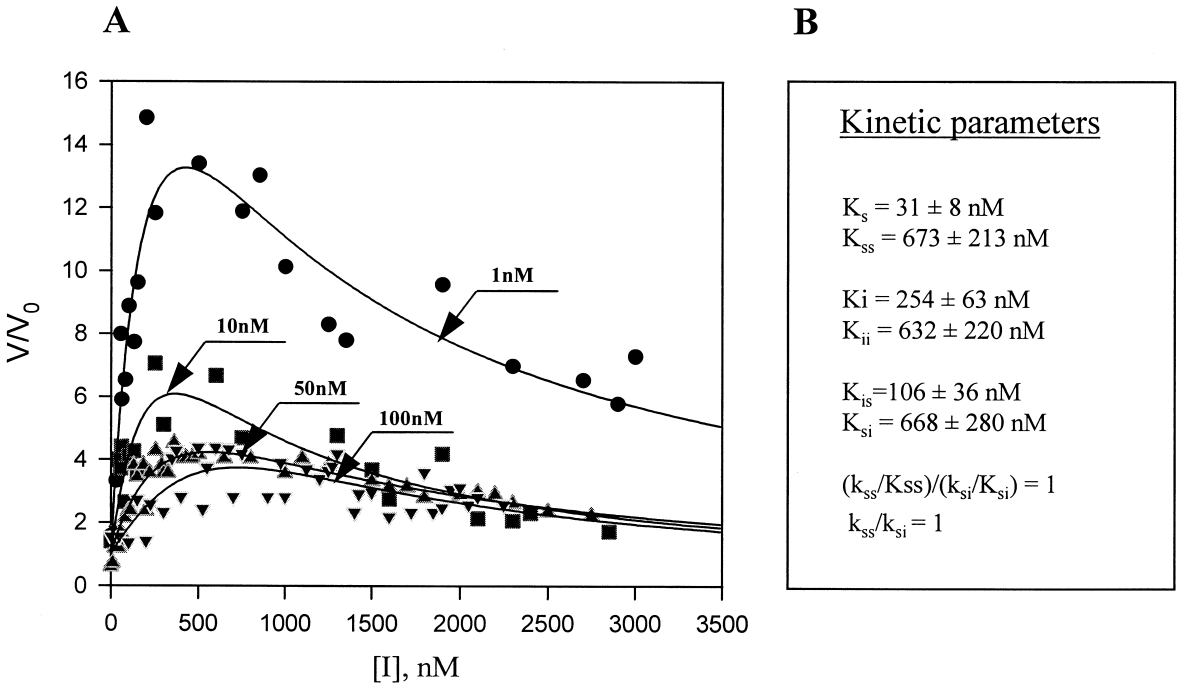


Fig. 5. The dependence of activation effect of substrate 71.1 cleavage by R·*Eco*RII on the activator 14.1 (I) concentration at different concentrations of the substrate. A: Experimental curves; (●) 1, (■) 10, (▲) 50 and (▼) 100 nM of 71.1. B: Calculated kinetic parameters.

II cutting of substrate 71.2 when only one strand of the DNA duplex was labeled (Fig. 3, lane 2).

Non-hydrolyzable pp bonds were introduced into this substrate, replacing one of the *Eco*RII scissile bonds in the upper or lower strand of the duplex (71.2pp1 and 71.2pp2 respectively). Introducing one pp bond at one site prevented cleavage of the opposite strand (phosphodiester bond) in the same recognition site but both strands of the second recognition site were cleaved efficiently (Fig. 6, lanes 1, 2 and 5, 6). Two pp bonds when they replaced two hydrolyzable bonds in one of the recognition sites decreased cleavage efficiency at the second (non-modified) recognition site of substrate 71.2pp3 (Fig. 6, lanes 3, 4).

4. Discussion

4.1. Competitive activation; kinetic justification of activation effect

Kinetic analysis showed that *Eco*RII is activated by a 14 bp substrate (activator) to rapidly cleave the 71 bp DNA duplex (substrate). The activation increased 71 bp substrate binding but not catalysis. Thus, the kinetic data fit the classical model for competitive activation in which the activator affects substrate binding [12]. According to this model two substrate molecules bind reversibly and interdependently at two identical DNA-binding sites of the enzyme. The kinetics for R·*Eco*RII on 71.1 substrate yielded a value of 0.0029 s^{-1} for k_{cat}. This value of k_{cat} appears to be lower than that for type II restriction endonucleases, which recognize only one target sequence in DNA [13]. In addition to *Eco*RII endonuclease, *Nae*I, *Bsp*MI and *Nar*I endonucleases were found to have recognition sites resistant to cleavage by the endonucleases [1]. Kinetic analysis indicated different mechanisms of activation for the enzymes [1,14]. *Eco*RII endonuclease showed kinetics similar to that for R·*Nar*I [1], which indicated that these enzymes have identical binding sites for substrate and activator.

The activation effect increases at decreasing substrate concentration (the maximum v/v_o value increased 4-fold when the substrate concentration was decreased from 100 nM to 1 nM) (Fig. 5). The activator exhibited a lower affinity for the enzyme (E_2) than did the substrate at the first step of the enzyme-substrate interaction (the K_s is about 60 nM in comparison with a K_i of 240 nM). This increased the activation effect on the theoretical curves (Fig. 2, mathematical modeling). The affinity of the second S molecule for E_2S decreases more dramatically (K_{ss}/K_s is 22) than the affinity of the second I molecule for E_2I (K_{ii}/K_i is 3). One can suppose that the difference

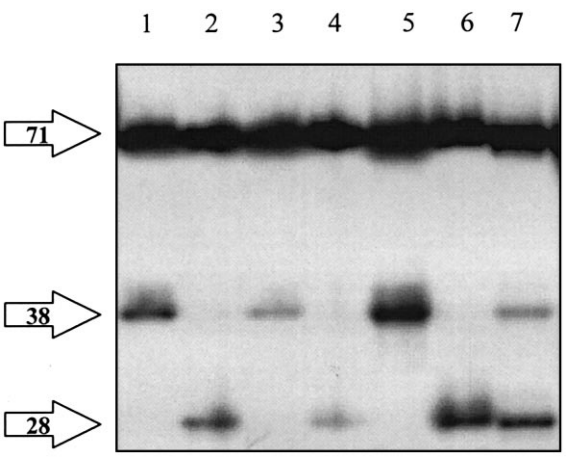


Fig. 6. Cleavage of pp bond-containing DNA duplexes with two recognition sites by R·*Eco*RII. Lanes 1 and 2: cleavage of 71.2pp1; lanes 3 and 4: 71.2pp3; lanes 5 and 6: 71.2pp2; lane 7: cleavage of 71.2; ^{32}P is in the upper (1, 3, 5 and 7) or lower (2, 4 and 6) strand of duplexes. Duplex concentration was 25 nM. Reaction time, 30 min. Figures on the left indicate the length of oligonucleotides.

in affinities is due to the different lengths of the substrates. The activation effect of 71.1 substrate hydrolysis thus seems to be due to the 6-fold higher affinity of substrate 71.1 to E_2I than to E_2S (K_{is} is about 104 nM in comparison with a K_{ss} of 632 nM). The experimental dependences of the activation effect both on the activator concentration and on the substrate concentration are in good agreement with those obtained by mathematical modeling. Taken together these data confirm the proposed kinetic scheme and the mathematical modeling approach could be proposed for prediction of kinetic prerequisite for the DNA duplexes to be used as activators.

4.2. *EcoRII cleaves one of the two coordinated recognition sites in one catalytic event*

Substrate 71.2 containing two *Eco*RII recognition sites is cleaved by the enzyme more effectively than that with a single recognition site. In this case *cis*-activation takes place. The short distance between two recognition sequences (5 bp) does not prevent the hydrolysis by a *cis*-activation mechanism. Because two products were observed after cleavage of each strand of substrate 71.2 by R·*Eco*RII and because the hydrolysis is provided by *cis*-interaction of two recognition sites it was proposed that the enzyme cuts only two phosphodiester bonds in one catalytic event (not four).

A two-site binding mechanism enables the enzyme to achieve DNA cleavage in two different ways: two phosphodiester bonds are cleaved within the same recognition site or within the different sites. Non-hydrolyzable pp bonds were introduced into this substrate, replacing one of the *Eco*RII scissile bonds in one of the strands of the duplex. Introducing one pp bond switches off the cleavage of the phosphodiester bond in the same recognition site (opposite strand), while both strands of the second recognition site are cleaved efficiently (at the same conditions). The data allow us to conclude that the enzyme cleaves rapidly two phosphodiester bonds of the same recognition site in one catalytic event.

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