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Inhibition of Eco RI action by polynucleotides. A characterization of the non-specific binding of the enzyme to DNA

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Abstract

The cleavage of the plasmid pBR322 by the restriction endonuclease Eco RI has been studied in the presence of various polynucleotides and the double stranded octanucleotide d-(GGAATTCC) in order to clarify whether there is a preferential interaction of Eco RI with DNA sequences other than -GAATTC-. The steady state kinetic analysis shows that all polynucleotides investigated with the possible exception of poly-dG•poly-dC inhibit the cleavage competitively with K_i values in the range of 10^{-4} to 10^{-5} [M nucleotides]. The K_i of d-(GGAATTCC) is $1.5 \cdot 10^{-6}$ [M nucleotides], indicating that the specific binding is approx. 2 orders of magnitude stronger than non-specific binding.

Introduction

The restriction endonuclease Eco RI very specifically cleaves double stranded DNA within the sequence GAATTC (for recent reviews cf. [1,2]). The enzyme also binds non-specifically to DNA not containing the recognition site. Non-specific binding to DNA seems to be a rather general phenomenon with proteins acting on DNA [3] and has in some instances been considered to increase the efficiency of the specific interaction [4,5,6].

Non-specific binding of Eco RI to DNA has been similarly regarded to be important for its enzymatic action [7], and has been investigated in detail recently [8,9]. Goppelt et al.[9] demonstrated by circular dichroism titrations employing oligonucleotides of defined sequence that in the absence of Mg^{2+} the non-specific interaction is of similar strength as the specific one, while in the presence of Mg^{2+} specific binding is favored by a factor of 50. Woodhead & Malcolm [8], on the other hand, proposed on the basis of protection experiments with phage DNA and synthetic polynucleotides that in the absence as well as in the presence of Mg^{2+} specific binding, which is supposed to occur at the sequence AATT, is 15000-fold stronger than non-specific binding. This would imply that contrary to the results of Goppelt et al. considerable specificity is exerted already in the binding of Eco RI to DNA.

In the present paper experimental results are reported which should help to resolve this apparent discrepancy and to characterize the non-specific binding more clearly. For this purpose, we have investigated the steady-state kinetics of the cleavage of the plasmid pBR 322, which contains one Eco RI site, in the presence of various natural and synthetic oligo- and polynucleotides. The inhibition constants are interpreted in terms of relative binding strengths.

Materials and Methods

Polynucleotides were obtained from Sigma Chemical Corp., München and from Boehringer, Mannheim. Calf thymus DNA was from Serva, Heidelberg. λ phage DNA used in the Eco RI assay was purchased from Miles Biochemicals, d-(GGAATTCC) from Collaborative Research, Waltham, Mass. Crude tRNA from E.coli was obtained from Boehringer. Culture media were obtained from Difco, Detroit, Mich. Tetracycline and chloramphenicol were from Boehringer. CsCl was purchased from Baker. Ethidium bromide was from Serva, Heidelberg. All other chemicals were reagent grade.

Purification of pBR 322 DNA

pBR 322 DNA was prepared from E.coli SK1592 containing the plasmid (courtesy of Dr. H. Mayer, GBF Stöckheim). A cleared lysate was prepared according to the method described by Clewell and Helinski [10]. The lysate was then prepared for gradient centrifugation by adding 0.954 g CsCl and 0.1 ml of a 10 mg/ml aqueous ethidium bromide solution to each ml of lysate and centrifuged at 110000 x g_{av} for 60-72 hours. The denser fluorescent band was removed from the gradient by side puncture with a hypodermic syringe. Ethidium bromide was removed from the DNA by repeated extractions with isopropanol. The plasmid was then dialyzed against 1 mM Tris x HCl pH 7.2, 0.1 mM EDTA. Usually the plasmid prepared by this method was approx. 90% superhelical and RNA-free. If the DNA was not satisfactorily pure after the first centrifugation, the banding procedure was repeated.

Purification of Eco RI

Eco RI was isolated from E.coli BS 5.Enzyme activity was assayed as described by Goppelt et al.[9] All buffers used throughout the isolation procedure contained 1 mM EDTA, 0.1 mM 1,4-dithioerythritol and 0.01 % (w/v) Lubrol. All operations were carried out at 4 °C.
750 g wet cell paste suspended in 1 l 0.05 M Tris x HCl pH 7.6 were passed

6 times through a Manton-Gaulin homogenizer at a pressure of 600 bar. Debris was removed by centrifugation at 30000 x g_{av} for 2 hrs. The supernatant was adjusted to pH 7.6 with NH_4OH and then diluted with H_2O to give the conductivity of a 0.2 M NaCl solution, before being applied to a DEAE-cellulose column (Whatman DE 52, 15 x 10 cm). The column was washed with 1 l 0.05 M Tris x HCl pH 7.6. To the combined effluents $(NH_4)_2SO_4$ was added to 55% saturation. After stirring for 1 hr, the precipitate was collected by 1/2 hr centrifugation at 30000 x g_{av}, suspended in 200 ml 0.05 M Tris x HCl pH 8.6, and dialyzed overnight against 10 l 0.05 M Tris x HCl pH 8.6, 0.1 M NaCl. The dialyzate was applied to a 5 x 20 cm Blue Sepharose column [11], from which the enzyme was eluted with a 2 x 2 l 0.2...0.7 M NaCl linear gradient in 0.05 M Tris x HCl pH 8.6. Enzymatically active fractions were combined and precipitated with $(NH_4)_2SO_4$ at 55% saturation. After 1 hr stirring, the precipitate was collected by centrifugation, suspended in 20 ml 0.2 M potassium phosphate pH 7.2 and dialyzed overnight against 5 l 0.02 M potassium phosphate pH 7.2. The dialyzate was loaded onto a 5 x 25 cm hydroxyapatite column [12]. Eco RI was eluted with a 2 x 1 l 0.2...0.4 M linear potassium phosphate gradient. The active fractions were combined and dialyzed against solid polyethylene glycol until the sample had a volume of approx. 5 ml. The turbid solution was dialyzed for 2 hrs against 1 l 0.05 M Tris x HCl pH 8.6, 0.1 M NaCl and then subjected to gel filtration on a 2.5 x 180 cm Sephadex AcA 44 column in 0.05 M Tris x HCl pH 8.6, 0.1 M NaCl. The pooled active fractions were diluted 1:1 with H_2O and applied to a 5 x 10 cm DNA cellulose column [13]. Eco RI was eluted with a 2 x 1 l 0.05... 0.5 M NaCl linear gradient in 0.05 M Tris x HCl pH 8.6. The active fractions were concentrated by air drying in a dialysis bag to a volume of approx. 5 ml. A subsequent dialysis against 0.05 M Tris x HCl pH 8.6, 0.1 M NaCl, 80% glycerol yielded an enzyme preparation stable for at least several months when stored at -40 °C. The yield of electrophoretically pure enzyme was 2.3 OD_{280} units, corresponding to 1.9 mg [14].

pBR 322 cleavage

All experiments were performed in 0.01 M Tris x HCl pH 7.2, 0.08 M NaCl, 0.02 M $MgCl_2$ at 37 °C. Reactions were stopped by addition of 20% (v/v) of a solution of 0.25 M EDTA pH 8.0, 0.2% (w/v) sodium dodecyl sulphate, 0.01% bromophenol blue and 25% (w/v) sucrose.
The cleavage products were separated on 1% agarose gels in a flat bed electrophoresis apparatus (15 x 20 cm, gel thickness 4-5 mm) at a field strength

of 4-6 V/cm. The electrophoresis buffer used was 40 mM Tris x acetate pH 8.2, 5 mM sodium acetate, 1 mM EDTA. The gels were stained in 1 µg/ml ethidium bromide in water at 37 °C under gentle shaking for 15 min, then photographed for 15 min under 354 nm UV light on Polaroid 665 positive/negative film through a Schott OG535 filter. For quantitative evaluation, the negatives were scanned with a gel scanner and the scans digitized and numerically integrated. A calibration had shown that peak areas thus obtained are proportional to DNA concentration within the range used. The relative amount of superhelical, open circular and linear DNA in each sample was normalized to the total DNA concentration present to correct for pipetting errors.

d-(GGAATTCC) cleavage

The cleavage of d-(GGAATTCC) by Eco RI was measured by following the release of d-GG. For this purpose, appropriate amounts of d-(GGAATTCC), which had been labeled at its 5'-OH end with γ-^{32}P-ATP as described in [15], were incubated at 20 °C with Eco RI in 0.01 M Tris x HCl pH 7.2, 0.08 M NaCl, 0.02 M $MgCl_2$. The reaction mixture was analyzed by homochromatography at 65 °C on DEAE - cellulose sheets (Polygram CEL 300 DEAE/HR-2/15, Macherey & Nagel). The homomix used was prepared according to Jay et al. [16] by hydrolysis of yeast RNA with 0.9 M KOH at 37 °C for 24 hrs. Radioactively labeled d-(GGAATTCC) and d-(GG) were located by autoradiography and quantitated by liquid scintillation counting.

Steady state kinetics for an Eco RI substrate containing non-specific sequences

To evaluate the influence of Eco RI binding to non-specific DNA outside the recognition sequence, it is assumed that the enzyme molecule can cleave the DNA only if it is either bound directly to the specific sequence or is able to reach its cleavage site before it dissociates again (e.g. by a linear diffusion mechanism [6]). Therefore, only enzyme molecules bound within a certain distance a/2 to both sides of the GAATTC site can cleave the DNA prior to dissociation. The remaining length of the DNA (ℓ - a) will take Eco RI molecules out of the reaction pathway, thereby effectively inhibiting the cleavage reaction.

Michaelis-Menten kinetics employing such a large DNA substrate will then give apparent K_M and v_{max} values that differ from the intrinsic ones. The overall shape of the rate vs. substrate concentration plot, however, remains the same as for a standard Michaelis-Menten mechanism. A short calculation

shows this:

If c_{DNA} is the total molar DNA concentration, the DNA contains one specific cleavage site and the length of the 'non-specific' DNA is ℓ times that of the region containing the specific site, one can write formally:

$$\begin{array}{lll} \text{Concentration of specific sites} & c_{spec} = & c_{DNA} \\ \text{Concentration of non-specific sites} & c_{non\text{-}spec} = & \ell \cdot c_{DNA} \end{array} \qquad /1/$$

Specific cleavage is described by a Michaelis-Menten mechanism:

$$\text{Eco RI} + \text{DNA}_{spec} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{Eco RI x DNA}_{spec} \xrightarrow{k_2} \text{Eco RI} + \text{DNA (cleaved)}$$

with $K_M = (k_{-1} + k_2)/k_1$ and in the steady state

$$c_{EcoRI \text{ x } DNA_{spec}} = c_{EcoRI} \cdot c_{spec} / K_M \qquad /2/$$

Non-specific binding is characterized by the dissociation constant K' :

$$\text{Eco RI} + \text{DNA}_{non\text{-}spec} \underset{}{\overset{K'}{\rightleftharpoons}} \text{Eco RI x DNA}_{non\text{-}spec}$$

$$c_{EcoRI \text{ x } DNA_{non\text{-}spec}} = c_{EcoRI} \cdot c_{non\text{-}spec} / K' \qquad /3/$$

From /1/,/2/,/3/ it follows that

$$c^{free}_{EcoRI} = c^{total}_{EcoRI} / (1 + c^{free}_{DNA} \cdot (1/K_M + \ell/K'))$$

With $c^{free}_{EcoRI} \ll c^{total}_{DNA}$, we may also replace c^{free}_{DNA} by c^{total}_{DNA}. For the overall reaction rate $v = k_2 \cdot c_{EcoRI \text{ x } DNA_{spec}}$, one then obtains:

$$v = \frac{k_2 \cdot c^{total}_{EcoRI}}{1 + \ell \cdot \frac{K_M}{K'}} \cdot \frac{c^{total}_{DNA}}{\frac{K_M}{1 + \ell \cdot K_M/K'} + c^{total}_{DNA}} \qquad /4/$$

This shows that both the intrinsic v_{max} and the intrinsic K_M are diminished by the same factor $(1 + \ell \cdot K_M/K')$, which is independent of substrate concentration. It is therefore justified to evaluate the steady state kinetics of Eco RI cleavage of a large DNA according to a classical Michaelis-Menten mechanism.

Results and Discussion

The cleavage of supercoiled pBR322 leads to linear DNA (Fig. 1). Under stea-

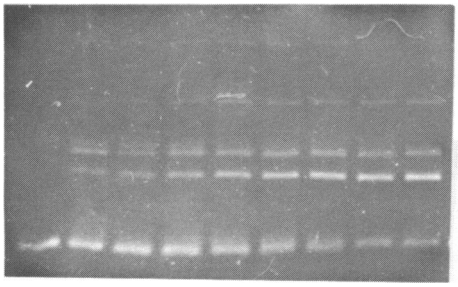

Fig.1 : Cleavage of pBR 322 by EcoRI under steady state conditions. 7.9 nM pBR322, 2 nM Eco RI; reaction times: 0',0.5',1',2',3',4',5',7',10'

— open circular pBR322
— linear "

— supercoiled "

dy state conditions at 37 °C, this reaction cannot be resolved in two steps. We have therefore taken the formation of linear DNA as a measure of overall reaction rate.

A double reciprocal plot of reaction rate versus substrate concentration yields a K_M of 5 [nM] and a turnover number of 1.8 [min^{-1}] (Fig. 2). Addition of oligo- or polynucleotides to the reaction mixture leads to a decrease of the apparent K_M, but does not affect v_{max} significantly (Fig.3). Table 1 gives inhibition data for various polynucleotides and for d-(GGAATTCC). Calf thymus DNA inhibits very efficiently; poly-dG x poly-dC, which is known to form abnormal heterocomplexes[17],is rather inefficient. tRNA also inhibits cleavage, which agrees with our previous observation that EcoRI is not only bound to immobilized polydeoxiribonucleotides, but also to polyribonucleotides[9]. Since addition of polynucleotides only af-

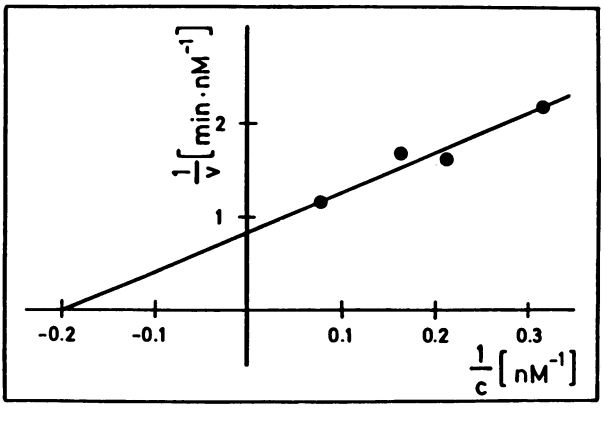

Fig.2: Double reciprocal plot of cleavage rate vs. plasmid concentration for pBR 322 cleavage by 0.65 nM Eco RI at 37 °C. Initial rates were obtained from the amount of linear plasmid DNA formed after 1 min.

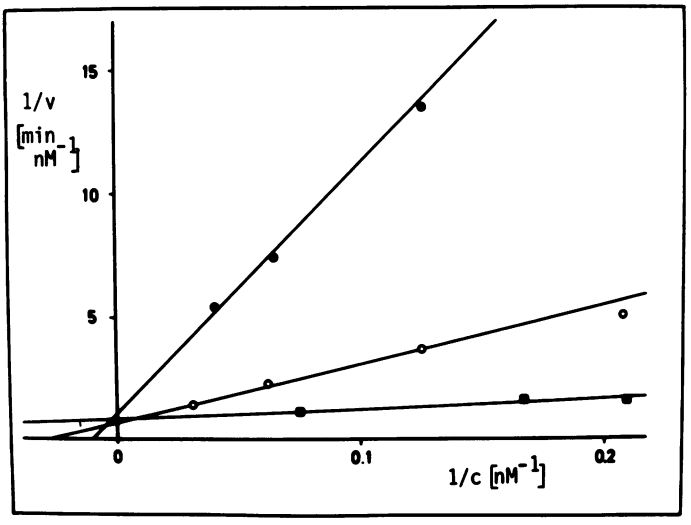

Fig.3 : pBR322 cleavage by 0.65 nM EcoRI at 37°C in the presence of 0 (■), 0.92 (○), 2.3 (●) mM crude E.coli tRNA (concentration given in nucleotides)

fects K_M, but not v_{max} significantly, the inhibition is competitive with an inhibition constant

$$K_i = c_i / (K_M^{app} / K_M^o - 1) ,$$

where c_i is the inhibitor concentration (for easier comparison given in units of nucleotides), K_M^o is the Michaelis constant in the absence and

Table 1 : Competitive inhibition of Eco RI by oligo- and polynucleotides

Inhibitor used	Concentration [µM Nucl.]	K_M^{app} [nM]	Turnover No. [min^{-1}]	K_i [µM Nucl.]
d-(GGAATTCC)	38	30	1.0	7.5
calf thymus DNA	300	60	1.2	27
poly-d(GC)	300	50	1.0	33
poly-d(AC)x poly-d(GT)	300	28	1.0	65
poly-d(AT)	380	18	2.0	150
"	760	31	2.0	150
$tRNA_{E.coli}$	920	36	1.4	150
	2300	100	0.8	120
poly-dIxpoly-dC	300	10	1.0	300
poly-dAxpoly-dT	375	10	1.8	375
poly-dGxpoly-dC	760	≥5	1.9	>1000

K_M^{app} in the presence of inhibitor.

Competitive inhibition implies that Eco RI non-specifically binds polynucleotides either at its active site or in such a way that enzyme-substrate interaction is prohibited. Because of the overall structural similarity of the substrate and the competitor we favor the idea that both share a binding site on the enzyme.

Although our data clearly show differences in K_i for different polynucleotides, a straightforward correlation between structure and strength of the non-specific binding cannot be derived. All we can say is that alternating purine-pyrimidine duplexes are bound more strongly than complementary homopolymer duplexes. It cannot be decided, however, whether it is the composition of the bases themselves that determines the binding strength or whether it is due to a sequence dependent variation in local conformation of the sugar-phosphate backbone as has been postulated for the binding of the lac-repressor to non-operator DNA [18].

Assuming a fast preequilibrium in the classical Michaelis-Menten scheme, inhibition experiments as reported in this paper as well as protection experiments [8] can be used to assess the relative affinity of Eco RI to its specific site and to non-specific sites. d-(GGAATTCC) contains the specific site; it is expected, therefore,to compete very efficiently with pBR322 for cleavage by Eco RI; this can be seen from Table 1. Since under the condi-

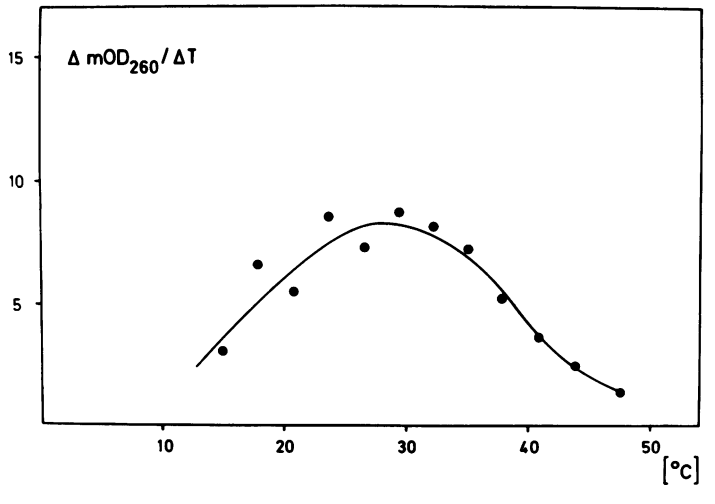

Fig. 4: Differential melting curve of 0.53 A_{260} units of d-(GGAATTCC) in 0.01 M Tris x HCl pH 7.2, 0.08 M NaCl, 0.02 M $MgCl_2$. The melting curve was determined according to Römer et al (21).

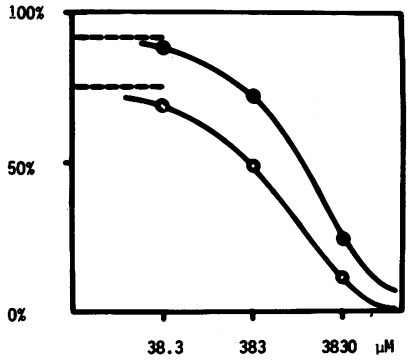

Fig.5: Cleavage of 0.5 µM d-(GGAATTCC) (≙ 4 µM Nucl.) by 26 nM Eco RI at 20 °C in the presence of increasing amounts of poly-d(AT). Rates expressed as relative amounts cleaved after 20 (○) and 40 (●) min. Poly-d(AT) concentration given in units of nucleotides. --- denotes the amount cleaved in the absence of poly-d(AT).

tions of the protection experiment only 20% of the oligonucleotide are double stranded (Fig. 4), the K_i of double stranded d-(GGAATTCC) can be extrapolated to be approx. 1.5 [µM]. This indicates that d-(GGAATTCC) is bound about two orders of magnitude more strongly than poly-d(AT).

Essentially the same result has been obtained by a different approach. The cleavage of d-(GGAATTCC) by Eco RI can be analyzed directly as described in Methods. As shown in Fig. 5, poly-d(AT) inhibits the cleavage of d-(GGAATTCC) half-maximal at a concentration 250 times as large as the d-(GGAATTCC) concentration. Under the conditions of this experiment, about 75% of the oligonucleotide are double stranded[9]. Consequently, this result is also in agreement with a two orders of magnitude stronger binding of d-(GGAATTCC). The fact that calf thymus DNA, which does contain Eco RI recognition sequences, does not compete nearly as effectively as d-(GGAATTCC), is reasonable since the binding of calf thymus DNA to Eco RI is dominated by the vast excess of non-specific sequences.

Our data, therefore, do not support the hypothesis of Woodhead & Malcolm [8] that the strong protection afforded to Eco RI by ΦX 174 RF and SV 40 DNA against chemical inactivation is due to a very strong binding of Eco RI to d-AATT sites. It seems more reasonable, on the basis of our experiments, to explain the results found in [8] by differences in the DNA primary structure other than d-GAATTC or d-AATT content, or possibly by tertiary structure differences [19,20]. The results reported in the present paper are in agreement with our previous results according to which Eco RI binds by two orders of magnitude more strongly to oligonucleotides containing the specific sequence as compared to other oligonucleotides. This finding can now be extended to polynucleotides.

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