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Transient cleavage kinetics of the Eco RI restriction endonuclease measured in a pulsed quench-flow apparatus: enzyme concentration-dependent activity change

J.Langowski, C.Urbanke, A.Pingoud and G.Maass

Zentrum Biochemie, Abt. Biophysikalische Chemie, Medizinische Hochschule, Karl-Wiechert-Allee 9, 3 Hannover 61, GFR

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SUMMARY

We report measurements of the cleavage rate of pBR 322 plasmid DNA by the restriction endonuclease Eco RI as a function of enzyme and DNA concentration. The reaction, which at high excess of enzyme over DNA occurs between 0.2 and 5 seconds, was studied by the means of a microprocessor controlled pulsed quench-flow apparatus.

Enzyme concentrations were between 1 and 100 nM with DNA concentrations being 3 to 6 nM (specific Eco RI sites). The catalytic constants for cleavage of the first and second phosphodiester bonds as measured at high enzyme concentration both have the same value of 0.35 sec^{-1} at 21 °C. At enzyme concentrations comparable to or less than DNA concentration, the rate of the first cleavage is proportional to enzyme concentration, while the second step is independent of concentration. At approx. 10 nM Eco RI endonuclease concentration, a rate increase shows up in both the first and the second cleavage. We suggest that this increase is due to the tetramerization reported by Modrich & Zabel[1], which occurs in this concentration range.

INTRODUCTION

The Eco RI restriction endonuclease is widely used in the analysis of DNA structure and in genetic engineering. Since Eco RI is a sequence specific endonuclease, it provides an excellent model for the study of highly specific protein-nucleic acid interactions in general. Although the specific recognition and cleavage of the DNA sequence 5'-GAATTC-3' by the enzyme has been the subject of several recent investigations[2-6], there is only very limited information about its mechanism as yet. Partly in lack of a suitable optical detection system for the association of Eco RI endonuclease to DNA and for the specific cleavage, the kinetics of the cleavage reaction have been

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investigated so far only under classical 'Michaelis-Menten' type conditions with low concentration of enzyme. 'Single-turnover' kinetics with excess of enzyme have been reported so far only once[1].

Eco RI endonuclease binds to specific and non-specific DNA sequences as shown by titrations monitoring the circular dichroism of the enzyme[7]; in addition, its nucleolytic action is inhibited by non-specific binding to a variety of polynucleotides[8]. 'Self-inhibition' which is a result of non-specific binding to the substrate DNA itself, cannot be detected under Michaelis-Menten conditions, as is shown in[8]. However, with fast kinetic measurements under saturating conditions of enzyme, one can overcome this problem.

The purpose of the work presented in this paper was to investigate the cleavage of DNA by the Eco RI endonuclease at enzyme concentrations ranging from those employed in Michaelis-Menten kinetics to saturation levels of enzyme. These experiments necessitated the use of a fast chemical quenching procedure which we carried out in a microprocessor controlled pulsed quench-flow machine recently constructed in this laboratory[9].

MATERIALS AND METHODS

Preparation of the enzyme

The enzyme was prepared from E. coli BS 5 (courtesy of Dr. H. Mayer, GBF Stoeckheim) as described in[8]. In SDS-polyacrylamide gel electrophoresis only one band could be detected, indicating that the enzyme was at least 95% pure. The concentration was determined using the extinction coefficient $E_{280}^{0.1\%} = 1.2$ given in[1].

DNA-PREPARATION

pBR322 plasmid DNA was transformed into E.coli strains SK 1592 or HB 101 according to the procedure described in[10]. SK 1592 preparations yield more plasmid DNA; however, approx. 10% of the superhelical DNA is obtained in the dimer form, which is not formed in recA$^-$ strains such as HB 101 (M. Zabeau, personal communication). Identical results were obtained with pBR322 from HB 101 and from SK 1592. Most of the measurements reported, therefore, have been carried out with pBR322 from E.coli SK 1592. SK 1592 cells were grown in L broth, HB 101

in L broth or in a minimal medium containing 1g NH_4Cl, 0.2 g $MgSO_4$, 1.5 g DH_2PO_4, 3.5 g NA_2HPO_4, 2 g glucose and 5 g casamino acids (Difco, Detroit, Mich.) per l of culture. Starter cultures (50 ml), containing an additional 40 µg/ml of tetracycline (Boehringer), were grown overnight from single colonies at 37°C and transferred to 2 l of medium in a shaker bath at 37°C. After a turbidity of 0.5 OD units at 550 nm was reached, 150 µg/ml chloramphenicol (Boehringer) were added and the culture incubated at 37°C overnight. Plasmid DNA was isolated from lysozyme - Triton X 100 - lysates of the cells by banding it twice in a CsCl-ethidium bromide gradient, followed by butanol extraction of the ethidium bromide and dialysis[8].

QUENCH-FLOW EXPERIMENTS

The microprocessor controlled quench-flow apparatus has two independently controllable pairs of syringes (syringes A/B and S/Q, resp.) that are driven by high-torque stepping motors (Fig. 1). For the cleavage experiments, syringe A contained pBR322 DNA in the standard buffer (0.01 M Tris x HCl pH 7.2, 0.08 M NaCl, 0.2 M $MgCl_2$), syringe B contained Eco RI endonuclease in the same buffer, syringe S contained water and

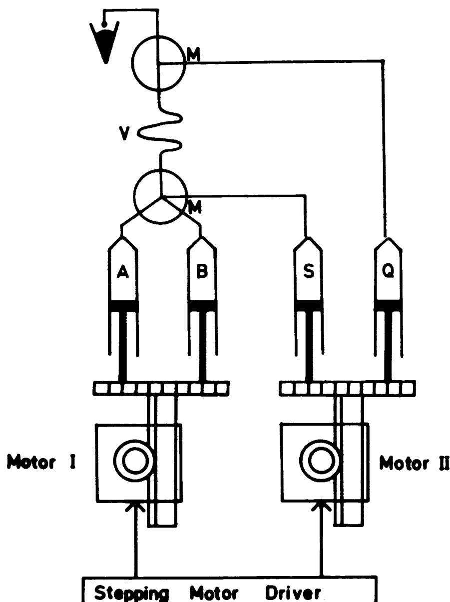

Fig.1: Schematic diagram of the pulsed Quench-Flow apparatus used in our experiments.
A: DNA solution, B: enzyme sol.
S: water, Q: 0.1 M EDTA
M: mixing chambers
V: reaction volume

Motor I and motor II are high-power stepping motors. These are driven by a microprocessor controlled driver (details of the machine and driver electronics given in[9]).

syringe Q the quenching solution (0.1 M EDTA pH 8.1). All experiments were done with the apparatus thermostated to 21°C. A reaction vessel with a volume of 50 µl (stainless steel capillary tube) was filled through a mixing chamber with equal volumes from syringes A and B. After a defined incubation time, the mixture was expelled from the reaction chamber by water from syringe S and mixed with the quenching solution from syringe Q. Samples were collected in Eppendorf microfuge tubes, lyophilized and resuspended in 20 - 50 µl of water. After adding 5 µl of 25 % sucrose/ 0.1 % bromophenol blue solution, the samples were subjected to electrophoreses on 1 % agarose gels. The gels were stained with ethidium bromide and the amount of DNA quantitated by photography[8].

ANALYSIS OF CLEAVAGE KINETICS

Under the conditions applied here, $c_{DNA} < K_m$ and c_{Enzyme}

c_{DNA},

both the first and the second cleavage steps follow apparent first order kinetics with respect to DNA concentration:

$$\text{superhelical (sh) DNA} \xrightarrow{k_1^{app}} \text{open circular(oc) DNA} \xrightarrow{k_2^{app}} \text{linear (li) DNA.} \quad (1)$$

One obtains k_1^{app} easily from a semi-logarithmic plot of the concentration of superhelical DNA (c_{sh}) vs. time. k_2^{app} is obtained from the integrated kinetic equations for (1):

$$c_{sh} = c_{sh}^{o} \cdot e^{-k_1 t} \; ; \qquad c_{oc} = c_{sh}^{o} \cdot \left(\frac{k_1}{k_2-k_1}\right) \cdot \left(e^{-k_1 t} - e^{-k_2 t}\right) \; ;$$

$$c_{li} = c_{sh}^{o} \cdot \left[1 + \left(\frac{1}{k_1-k_2}\right) \cdot \left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right) \right] \qquad (2)$$

where k_1^{app} and k_2^{app} have been denoted k_1 and k_2 for convenience; c_{oc} is the concentration of the open circular form, c_{li} of the linear form and c_{sh}^{o} of the superhelical form at t=0.

These equations yield for the maximum concentration of the intermediate open circular form c_{oc}^{max} :

$$c_{oc}^{max} = c_{sh}^{o} \cdot \left(\frac{k_1}{k_2}\right)^{\left(\frac{1}{1 - k_1/k_2}\right)} \qquad (3)$$

Since all preparations of superhelical DNA usually contain a varying proportion f of non-specifically nicked open circular DNA, eq. (3) has to be modified accordingly:

$$c_{oc}^{max} = c_{sh}^{o} \cdot \left[\left(\frac{k_1}{k_2}\right) + f \cdot \left(1 - \frac{k_1}{k_2}\right)\right]^{\left(\frac{1}{1 - k_1/k_2}\right)} \qquad (4)$$

k_2^{app} was then obtained from eq. (4).

RESULTS AND DISCUSSION

The cleavage of pBR322 plasmid DNA by EcoRI endonuclease proceeds in two steps: First, the superhelical DNA is nicked in one strand to yield the open circular form, which is then cleaved a second time to yield the linear form. Phenomenologically, one can distinguish between to limiting cases: at lower enzyme concentrations, the reaction proceeds directly to the linear form (Fig. 2a); at higher endonuclease concentrations, a significant increase in the intermediate open circular form is seen (Fig. 2b). Thus, at low enzyme concentrations, the first cleavage seems to be the rate limiting step while under saturation conditions (excess enzyme) both cleavages proceed with comparable rates. An identical result was obtained in cleavage experiments with supercoiled pMB 9 plasmid DNA (data not shown), indicating that our conclusions are valid not only for pBR322. Michaelis-menten kinetics of the EcoRI endonuclease catalyzed cleavage of ColE1 and pMB 9 have also been reported in [1] and [2], resp. While the ColE1 experiments support our conclusions, the pMB 9 experiments do not, since in [2] both cuts have been reported to proceed with comparable rates even at low enzyme concentrations. As a tentative explanation we suggest that this discrepancy is due to different enzyme preparations.

The concentration dependence of k_1^{app} and k_2^{app} is shown in Fig. 3. Two major facts result from these data: First, saturation of

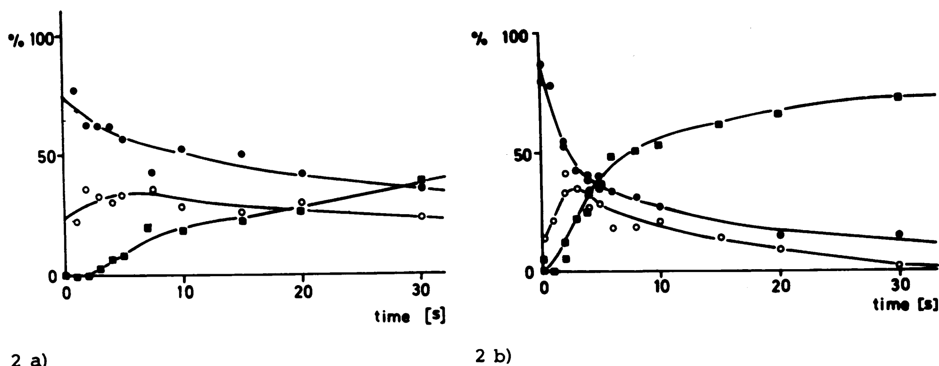

Fig.2: Cleavage of 3 nM pBR322 DNA by a) 13.4, b) 21 nM EcoR1 endonuclease.
● superhelical DNA o open circular DNA ■ linear DNA

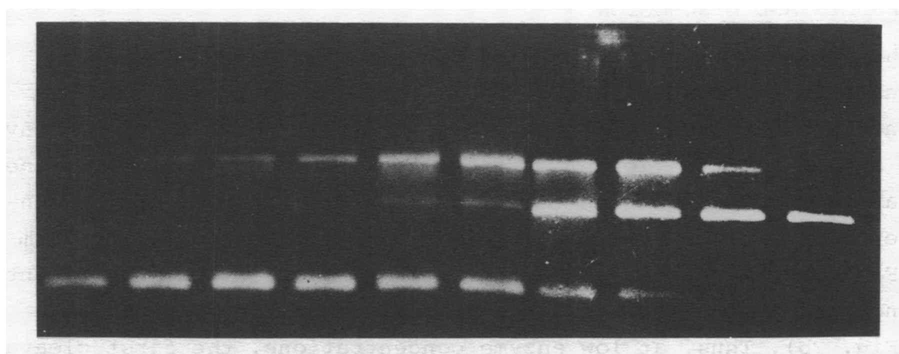

Fig. 2c: Gel pattern of a typical quench-flow experiment. Cleavage of 6nM pBR322 DNA from E.coli HB101 by 40 nM EcoR1 endonuclease. Reaction times were 0.02, 0.3, 0.6, 1, 1.2, 1.5, 3, 5, 7.5, and 10 sec. Buffer conditions for all experiments are given in the Materials section.

cleavage rate is achieved only at a high excess of enzyme over substrate ($c_{Enzyme} \simeq 20\ c_{DNA}$). Second, k_1^{app} depends on enzyme concentration in a non-linear fashion: The first cleavage is rate limiting at low enzyme concentrations; in this range the enzyme activity follows 'normal' Michaelis-Menten kinetics, i.e., activity is proportional to concentration. However, at an enzyme concentration of approx. 10 nM, the cleavage rate begins to increase more than linearly with enzyme concentration until it reaches a saturation level of 0.35 [sec $^{-1}$] at approx. 50 nM endo-

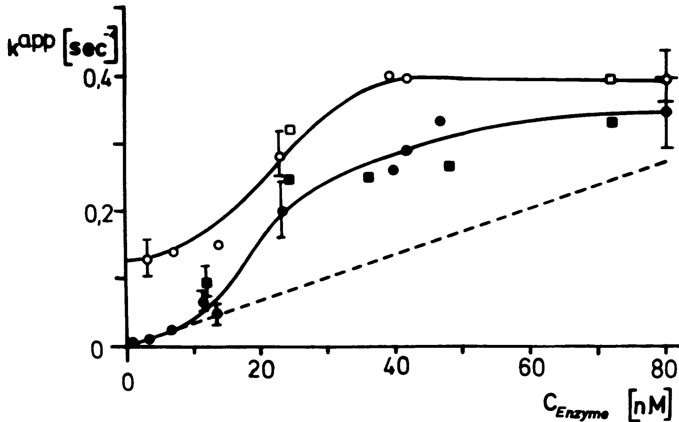

Fig.3: Dependence of k_1^{app} and k_2^{app} on enzyme concentration. Closed symbols: k_1, open symbols: k_2. Circles denote experiments performed at 3 nM DNA concentration, squares at 6 nM. The dashed line indicates the behavior of k_1 to be expected without the activity increase reported here.

nuclease. k_2^{app} equals k_1^{app} under these conditions. At low enzyme concentrations, however, k_2^{app} stays constant at 0.1 $[sec^{-1}]$.

The fact that the cleavage rate reaches its maximum only at a large excess of enzyme over substrate can be easily explained in the light of the previous finding that EcoRI endonuclease binds non-specifically to a variety of polynucleotides [7,8]. Therefore, enzyme molecules that are bound to the DNA distant from the specific cleavage site will be able to cleave only after dissociation and reassociation. Thus, the effective concentration of free endonuclease in the neighborhood of the specific site is reduced and maximum rate is reached only at higher concentrations. It still remains possible, however, that enzyme molecules diffuse linearly along the DNA and reach their specific site without dissociating and reassociating. This then would lead to an increase in the cleavage rate, i.e. an effective enlargement of the specific site. From our present data we cannot make any quantitative estimate of such a linear diffusion range, since this requires a much more detailed knowledge of the parameters for specific and non-specific binding than presently available.

The second conclusion we draw from our data is that there is

a concentration dependent activity change in the range of 10-20 nM EcoRI endonuclease. This activity change could be associated with the dimer to tetramer transition of the enzyme reported to take place at 10 to 100 nM dimeric enzyme[1]. Our data are in agreement with a model in which the activity for the first cleavage increases by a factor of 3-10 for the tetrameric form. This is supported by the simultaneous increase of k_2^{app} by a factor of 3. The activity increase is not dependent on DNA concentration between 3 and 6 nM DNA (Fig. 3), which is in the range of the K_m of the enzyme for pBR322 [8]. Therefore, our data indicate that the dimer to tetramer transition is not influenced by the presence of DNA to a measurable extent.

The activity change seems to be dependent on the type and size of substrate DNA used since experiments with the double stranded octanucleotide, d-GGAATTCC, do not show this activity change[11].

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