The *EcoRI* restriction endonuclease with bacteriophage λ DNA

Equilibrium binding studies

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The *EcoRI* restriction endonuclease was found by the filter binding technique to form stable complexes, in the absence of Mg^{2+} , with the DNA from derivatives of bacteriophage λ that either contain or lack *EcoRI* recognition sites. The amount of complex formed at different enzyme concentrations followed a hyperbolic equilibrium-binding curve with DNA molecules containing *EcoRI* recognition sites, but a sigmoidal equilibrium-binding curve was obtained with a DNA molecule lacking *EcoRI* recognition sites. The *EcoRI* enzyme displayed the same affinity for individual recognition sites on λ DNA, even under conditions where it cleaves these sites at different rates. The binding of the enzyme to a DNA molecule lacking *EcoRI* sites was decreased by Mg^{2+} . These observations indicate that (a) the *EcoRI* restriction enzyme binds preferentially to its recognition site on DNA, and that different reaction rates at different recognition sites are due to the rate of breakdown of this complex; (b) the enzyme also binds to other DNA sequences, but that two molecules of enzyme, in a different protein conformation, are involved in the formation of the complex at non-specific sequences; (c) the different affinities of the enzyme for the recognition site and for other sequences on DNA, coupled with the different protein conformations, account for the specificity of this enzyme for the cleavage of DNA at its recognition site; (d) the decrease in the affinity of the enzyme for DNA, caused by Mg^{2+} , liberates binding energy from the DNA–protein complex that can be used in the catalytic reaction.

A large number of nucleases can cleave duplex DNA at the sequence 5' -G-A-A-T-T-C- 3'. But unique among these enzymes at the present time is the *EcoRI* restriction endonuclease (EC 3.1.23.13) for, unlike the non-specific nucleases that can cleave DNA at this or any other sequence, the *EcoRI* enzyme cannot readily cleave DNA at any alternative sequence (Hedgpeth *et al.*, 1972). At high pH, or in the presence of Mn^{2+} , the *EcoRI* enzyme will cleave DNA at certain sites in addition to the above recognition sequence (Polisky *et al.*, 1975; Hsu & Berg, 1978), but at near neutral pH and in the presence of Mg^{2+} , cleavages of DNA at sequences apart from the recognition site are extremely slow. [The observation of Bishop (1979) that the *EcoRI* restriction enzyme can cleave one strand of the duplex DNA of a particular plasmid at the sequence of 5' -G-A-A-T-T-A- 3' does not appear to be a general property of this enzyme; this sequence is present at three locations in the replicative form of the DNA from bacteriophage ϕ X174 (Sanger *et al.*,

Abbreviation used: Tes, 2-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino]ethanesulphonic acid.

1978) and this DNA is attacked very slowly by *EcoRI*; see Fig. 6 below.]

However, no enzyme can be infinitely specific for a single substrate, so even under conditions where the *EcoRI* restriction endonuclease has a very marked preference for its recognition site, the enzyme must still display a finite activity against other DNA sequences. We have examined the specificity of the *EcoRI* restriction enzyme by measuring its binding to DNA molecules derived from bacteriophage λ (Murray & Murray, 1974) that either contain or lack *EcoRI* recognition sites. This has been achieved with the filter binding technique; Modrich (1979) reported that a stable complex between the restriction enzyme and DNA could be trapped on nitrocellulose filters. As applied to DNA–protein interactions, this technique relies on the ability of linear double-stranded DNA molecules to pass through a nitrocellulose membrane filter whereas proteins stick to the filter. Thus, if a mixture of the protein and radioactively labelled DNA is drawn through a filter of this type, the radioactivity retained on the filter is directly pro-

portional to the number of DNA molecules to which at least one molecule of protein has bound (Riggs *et al.*, 1970). Among a wide range of other protein–nucleic acid interactions, the filter binding method has been used with class I restriction endonucleases (Yuan *et al.*, 1975); the class I enzyme, *EcoK*, formed a complex with DNA that could be trapped on a filter only in the presence of all of the cofactors required for class I endonuclease activity (*S*-adenosylmethionine, ATP and Mg^{2+}) and the DNA had to contain the appropriate unmodified recognition site.

We report here that, in the absence of Mg^{2+} , the class II restriction endonuclease *EcoRI* will form stable complexes that can be trapped on filters with DNA molecules that contain and that lack *EcoRI* recognition sites; Mg^{2+} is the only cofactor required by a class II restriction enzyme. The effect of Mg^{2+} upon the binding of the enzyme to DNA was also studied with a DNA molecule lacking *EcoRI* recognition sites. A preliminary account of this work has been published (Halford, 1980). Other experimental techniques have now revealed the ability of the *EcoRI* restriction enzyme to bind to any DNA (Woodhead & Malcolm, 1980), and to synthetic oligonucleotides (Goppelt *et al.*, 1980).

Materials and methods

All materials and methods, except for the filter binding technique, have been described (Halford *et al.*, 1979, 1980). These include the DNA from all the derivatives of bacteriophage λ used here (Murray & Murray, 1974; see Fig. 1 of Halford *et al.*, 1980). The definitions of the terms k_b , k_2 and K_m^* , used in this paper to describe the kinetics of the *EcoRI*-catalysed cleavage of DNA, are also retained from the previous paper (eqns. 2, 3 and 7 of Halford *et al.*, 1980).

Filter binding experiments were carried out by a method similar to that employed by Riggs *et al.* (1970). Nitrocellulose membrane filters (from Sartorius, Göttingen, W. Germany; type 11306, 0.45 μ m pore size, 2.5 cm diameter) were boiled against several changes of distilled water and then stored under water at 4°C until required. Filter binding experiments were conducted on a standard filter holder at a vacuum of 3 cmHg (4 kPa) to yield a flow rate of 3 ml/min. For each experiment, a 0.5 ml sample of the buffer used in the subsequent enzyme/DNA mixture (typically 50 mM-Tes/50 mM-NaCl/1 mM-EDTA, pH 7.0) was first drawn through the filter; next, 0.95 ml of the mixture of *EcoRI* restriction enzyme and [3 H]DNA in the same buffer was filtered; finally, 0.5 ml of the buffer was drawn through after the passage of the enzyme/DNA mixture. The filter was dried at 110°C and the radioactivity retained on the filter was determined by

scintillation counting after the addition of the filter to 4 ml of toluene containing 6 g of 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole/litre. For some experiments, the mixture of enzyme and DNA in the Tes/NaCl buffer also contained $MgCl_2$; here, the equilibration of the filter used the same Tes/NaCl/ $MgCl_2$ solution as the subsequent binding mixture, but the washing of the filter after the enzyme/DNA mixture employed a solution containing the same concentration of Tes and NaCl as before but now 15 mM-EDTA instead of $MgCl_2$.

Mixtures of the *EcoRI* restriction enzyme and DNA were prepared for filter binding experiments by dispensing into vials 0.98 ml samples from one solution containing both unlabelled DNA and [3 H]DNA from the same bacteriophage λ derivative to yield the requisite specific radioactivity (generally 1×10^6 c.p.m./pmol). Then 20 μ l of *EcoRI* restriction enzyme at the appropriate dilution was added to each sample; the enzyme was stored in 10 mM- K_2HPO_4 /200 mM-NaCl/10 mM- β -mercaptoethanol/50% (v/v) glycerol/0.2% Triton X-100/0.01% gelatin, pH 7.5, and dilutions of the enzyme were made in the same solution. (We added a fixed volume of enzyme as one of the components of the enzyme buffer, probably gelatin, interfered with the filter retention of enzyme–DNA complexes when present at higher concentrations than that used here.) The mixtures of enzyme and DNA were incubated for about 10 min at 22°C and then filtered through the nitrocellulose membrane filters at room temperature. (The amount of DNA retained on the filter at all concentrations of enzyme tested was found to be invariant with time between 1 and 30 min after preparing the mixtures.)

For each set of experiments carried out with one DNA solution, background values for the radioactivity retained on the filters were obtained by filtering samples of the DNA solution to which 20 μ l of the enzyme buffer had been added. In the absence of enzyme about 2% of the total radioactivity was retained. The values given below for the radioactivity retained after filtering enzyme–DNA mixtures have already been corrected for the background values. If the filters were not washed with 0.5 ml of buffer after the DNA solution, the background readings were about 10% of the input radioactivity. In controls, the wash step was omitted from some filter binding experiments but, as had been noted previously (Riggs *et al.*, 1970), no differences were found between these experiments and those where a wash step was included, apart from the background values; both procedures yielded the same value for the equilibrium dissociation constant. Hence washing the filters with a fixed small volume of buffer did not elute enzyme–DNA complexes from the filter. At very high concentrations of enzyme ($[E_0] \gg K_D$), we found that

every batch of filters retained greater than 95% of radioactivity from the [^3H]DNA; this observation differs from that of Riggs *et al.* (1970) where considerably less than 100% of the input radioactivity was maximally retained and where the maximal retention varied between batches of filters (Lin & Riggs, 1972). We have employed filters from a different manufacturer to those used by Riggs *et al.* (1970).

Evaluations of the equilibrium dissociation constant (K_D) for the *EcoRI* restriction enzyme and the DNA from each of the derivatives of bacteriophage λ were carried out by titrating a fixed concentration of the requisite λ DNA at 30 pM with excess enzyme in the range 57 pM–85 nM. Under these conditions the equilibrium:

should yield a linear Eadie plot of

$$[\text{ED}] = [\text{D}_0] - \frac{[\text{ED}]}{[\text{E}_0]} \cdot K_D \quad (2)$$

where $[\text{E}_0]$ and $[\text{D}_0]$ are the total concentrations of enzyme and DNA present in the mixture; $[\text{ED}]$ is the equilibrium concentration of the enzyme–DNA complex which here is taken to be directly proportional to the radioactivity on the filter. Hence the Eadie plot of (radioactivity retained on filter) against (radioactivity/ $[\text{E}_0]$) is linear, has a gradient equal to $-K_D$ and an ordinate intercept corresponding to the radioactivity on the filter when all of the DNA is retained. This analysis demands that the concentrations of the complex, $[\text{ED}]$, is negligible compared with the total enzyme concentration, $[\text{E}_0]$; this condition was maintained throughout these titrations by ensuring that either $[\text{E}_0] \gg [\text{D}_0]$ or $[\text{E}_0] \ll K_D$ in each binding mixture so that $([\text{E}_0] - [\text{ED}])$ may be equated to $[\text{E}_0]$. Some titrations of a fixed DNA concentration with varied enzyme concentrations were carried out with the DNA from $\lambda 416$ (5) at 3 nM so that under the conditions of these experiments (50 mM-Tes/50 mM-NaCl/1 mM-EDTA, pH 7.0), the DNA concentration was larger than the K_D value; these binding curves were analysed by the method of Stinson & Holbrook (1973) and they yielded the same value for K_D at 0.5 nM as did the titrations at 30 pM-DNA under otherwise identical conditions. Hence the values of K_D are independent of the DNA concentrations.

All molarities of the *EcoRI* restriction endonuclease are in terms of the dimeric enzyme of mol.wt. 58 000. However, at the very highest enzyme concentrations employed in these studies, it is possible that a portion of the *EcoRI* enzyme may aggregate to the tetrameric form (Modrich & Zabel, 1976). But data obtained at very high enzyme concentrations, when $[\text{E}_0] \gg K_D$, do not contribute significantly to the evaluation of K_D .

Results and discussion

Filter binding curves

We have employed the DNA from three derivatives of bacteriophage λ (Murray & Murray, 1974): $\lambda 395$ (0) from which all *EcoRI* recognition sites have been removed by mutations; $\lambda 401$ (2) which possesses only *srl* 2 out of the five *EcoRI* recognition sites on wild-type λ DNA; $\lambda 416$ (5) which carries only *srl* 5; (see Fig. 1 of Halford *et al.*, 1980). Under the experimental conditions used for equilibrium binding studies, but with the addition of 15 mM-MgCl₂, the *EcoRI* restriction endonuclease cleaved both strands of the DNA from $\lambda 401$ (2) and $\lambda 416$ (5) at only the recognition sites on these DNA molecules whereas the DNA from $\lambda 395$ (0) did not incur any detectable level of double strand cleavages at the highest enzyme concentrations used here. In the presence of 1 mM-EDTA and the absence of added MgCl₂, none of the DNA molecules from $\lambda 395$ (0), $\lambda 401$ (2) or $\lambda 416$ (5) received double-strand cleavages from the *EcoRI* restriction enzyme.

However, we found that if a mixture of the *EcoRI* restriction endonuclease and [^3H]DNA from any of these λ derivatives was drawn through a nitrocellulose membrane filter in the absence of Mg²⁺, a portion of the radioactivity from the [^3H]DNA was retained on the filter. For each DNA species, the amount of [^3H]DNA retained on the filter was measured at a fixed concentration of that DNA but over a range of enzyme concentrations (Fig. 1a). In the absence of the *EcoRI* enzyme, very little radioactivity was retained on the filter but progressively more was retained as the enzyme concentration was raised until at high enzyme concentrations, close to 100% of each species of [^3H]DNA remained bound to the filters.

Since the retention of the [^3H]DNA on the filters must be due to the formation of a complex between DNA and the *EcoRI* restriction endonuclease, and since no hydrolysis of the DNA takes place in the absence of Mg²⁺, the observations in Fig. 1(a) were analysed as equilibrium-binding curves (Fig. 1b). With the DNA from both $\lambda 401$ (2) and $\lambda 416$ (5), but not from $\lambda 395$ (0), the titration of the DNA by the enzyme followed a hyperbolic binding curve up to the saturation of the DNA. Analysis of the data obtained with either $\lambda 401$ (2) or $\lambda 416$ (5) DNA by using eqn. (2) yielded linear Eadie plots (Fig. 1b) and thus a single equilibrium dissociation constant (K_D) is sufficient to describe the entire binding curve with either of these DNA molecules. In the particular experiments shown in Fig. 1(a), the *EcoRI* restriction enzyme had a slightly higher affinity for the DNA from $\lambda 401$ (2), where a value of 0.7 nM was obtained for K_D , than for the DNA from $\lambda 416$ (5), for which a K_D of 1.1 nM was found; however, repetition of these experiments indicated that, within

Fig. 1. Filter binding curves

(a) Samples (1.0 ml) containing the concentration of *Eco*RI restriction endonuclease indicated by $[E_0]$ and 30 μM - ^{3}H -DNA from one of the derivatives of bacteriophage λ specified below in 50 mM-Tes/100 mM-NaCl/1 mM-EDTA, pH 7.0, were individually drawn through a nitrocellulose membrane filter and the radioactivity retained on each filter was measured: $\circ$, DNA from λ 401 (2); $\bullet$, DNA from λ 416 (5); $\square$, DNA from λ 395 (0). The specific radioactivity of the ^{3}H -DNA from either λ 395 (0), λ 401 (2) or λ 416 (5) was adjusted so that 1.0 ml of 30 μM -DNA produced 3.1×10^4 c.p.m. in our counting system. (b) The variations between the concentration of the *Eco*RI restriction endonuclease, $[E_0]$, and the amount of the radioactivity retained on nitrocellulose membrane filters, after filtering mixtures of the enzyme and 30 μM - ^{3}H -DNA from one of the following derivatives of bacteriophage λ in 50 mM-Tes/100 mM-NaCl/1 mM-EDTA, pH 7.0, were analysed by Eadie plots (eqn. 2): $\circ$, DNA from λ 401 (2); $\bullet$, DNA from λ 416 (5); $\square$, DNA from λ 395 (0).

the experimental limitations, the enzyme had the same affinity for each DNA. In contrast, a different form of equilibrium binding curve was noted with the DNA from λ 395 (0); the binding of the *Eco*RI restriction enzyme to this DNA followed a sigmoidal binding curve rather than a hyperbola (Fig. 1a) and analysis of these data produced a markedly

non-linear Eadie plot (Fig. 1b). Thus the binding of *Eco*RI enzyme to λ 395 (0) DNA cannot be reconciled to a simple reaction with only one equilibrium constant (eqn. 1). The sigmoidal binding curve and the direction of the curvature in the Eadie plot (Fersht, 1977) both demonstrate strongly that the DNA molecules from λ 395 (0) exhibit

positive co-operativity with respect to the binding of the *EcoRI* restriction enzyme.

We consider that the most probable cause for the different forms of binding curves is that the DNA from λ 395 (0) lacks any *EcoRI* recognition sites, whereas the DNA from λ 401 (2) and λ 416 (5) each contain a single *EcoRI* recognition site. Consequently, the mechanism by which the enzyme binds to DNA that contains a recognition site differs from the mechanism of binding to DNA without recognition sites. [We have examined the possibility that the sigmoidal binding curve with λ 395 (0) DNA is due to an artefact of the system. However, the sigmoidal binding curve cannot be due to an artefactual property of the enzyme, such as its inactivation at high dilution, for this would result in sigmoidal binding curves being observed with all species of DNA used here. It is conceivable that the preparations of λ 395 (0) DNA contain an inhibitor of the binding reaction that is not present in the other DNA preparations; however, we have rejected this possibility because the same results were obtained with four separate preparations of the DNA from λ 395 (0), all of which were isolated by a procedure identical with that employed with the other λ derivatives.]

Binding at *EcoRI* recognition sites

The observations in Fig. 1 demonstrate directly that the binding of the *EcoRI* restriction enzyme to λ DNA derivatives containing an *EcoRI* site must be occurring at that recognition site. The DNA molecules from λ 395 (0), λ 401 (2) and λ 416 (5) are very similar. Thus, if the binding of the enzyme to the DNA from λ 401 (2) or λ 416 (5) had been taking place at random DNA sequences away from the single *EcoRI* site present in these two DNA species, no difference would have been observed between these binding curves and that found with the DNA from λ 395 (0). As a major difference was observed between the forms of these binding curves, we conclude that the complex between the *EcoRI* restriction enzyme and the DNA from λ 401 (2) that is detected by the filter binding assay has been formed at *srI* 2, the only *EcoRI* recognition site that is present in this DNA. Likewise, the filter binding assay with the DNA from λ 416 (5) measures the formation of the complex with the enzyme at only *srI* 5 from λ DNA.

We have described some kinetic experiments on the reactivity ($k_{\text{cat.}}/K_m$) of the *EcoRI* restriction enzyme towards both *srI* 2 on the DNA from λ 401 (2) and *srI* 5 on the DNA from λ 416 (5) (Halford *et al.*, 1980). We noted that the *EcoRI* enzyme had the same reactivity towards its recognition sites on these two DNA molecules in reaction mixtures containing NaCl concentrations below 50 mM, but that higher NaCl concentrations caused a decreased reactivity

of the enzyme towards λ 401 (2) DNA and an enhanced reactivity of the enzyme towards λ 416 (5) DNA. In order to determine whether these kinetic differences were due to alterations in the affinity of the enzyme for each recognition site, a series of filter binding experiments similar to those in Fig. 1 were carried out with the *EcoRI* restriction enzyme and the DNA from both λ 401 (2) and λ 416 (5) in the presence of a range of NaCl concentrations. With both DNA species and at every NaCl concentrations tested, the titrations of each fixed DNA concentration yielded hyperbolic equilibrium binding curves against the varied enzyme concentrations; for each titration a single value for K_D was determined from the linear Eadie plot. The variation with the NaCl concentration in the K_D values for the binding of the *EcoRI* enzyme to either λ 401 (2) or λ 416 (5) DNA is shown in Fig. 2. Throughout the range of NaCl concentrations tested, no significant differences were found at any one ionic strength between the K_D values with either DNA.

Thus the equilibrium binding data contrasts with the kinetic data of Halford *et al.* (1980) in which the variation of $k_{\text{cat.}}/K_m$ with NaCl concentration for the reaction on λ 401 (2) DNA deviated from that on λ 416 (5) DNA. With the DNA from λ 401 (2), the dependence upon the NaCl concentration for both the K_D and the $k_{\text{cat.}}/K_m$ values were equivalent; both the minimum value of K_D and the maximum value of

Fig. 2. NaCl dependency

Equilibrium dissociation constants, K_D , were evaluated from filter binding experiments with the *EcoRI* restriction endonuclease and the DNA from either λ 401 (2) (○) or λ 416 (5) (●) in 50 mM-Tes/1 mM-EDTA, pH 7.0 and the NaCl concentration indicated.

$k_{\text{cat.}}/K_m$ were found at 50mM-NaCl, and the increments in K_D at other ionic strengths were matched quantitatively by decreases in the $k_{\text{cat.}}/K_m$ values. However, for the DNA from λ 416 (5), no such correlation was found; the enhanced reactivity of the *Eco*RI restriction enzyme observed at 150mM-NaCl with this DNA was not accompanied by any decrease in the K_D value between the enzyme and λ 416 (5) DNA at this salt concentration. A mechanism for the *Eco*RI restriction endonuclease has been described by Halford *et al.* (1980) that can account for this apparent discrepancy on the basis of the relative magnitudes of the rate constants for the dissociation of the enzyme from its recognition site on DNA [postulated to be the same from λ 401 (2) and λ 416 (5) DNA] and the rate constants for the cleavage of the enzyme-bound DNA [postulated to be larger for λ 416 (5) DNA than for λ 401 (2) DNA].

Fig. 3. Interactions with recognition site

Left-hand ordinate, O: samples (1.0 ml) containing the concentration of *Eco*RI restriction endonuclease indicated by $[E_0]$ and 30 pmol $[\text{H}]$ DNA from λ 416 (5), at a specific radioactivity of 2×10^6 c.p.m./pmol, in 50 mM-Tes/50 mM-NaCl/1 mM-EDTA, pH 7.0, were individually drawn through a nitrocellulose membrane filter and the radioactivity retained in each filter was measured. Right-hand ordinate, ●: first-order rate constants, k_b , were evaluated from the time course for product formation (after the cleavage of both DNA strands at the recognition site) in reactions at 22°C containing the concentrations of *Eco*RI restriction endonuclease indicated by $[E_0]$ and 0.5 nM $[\text{H}]$ DNA from λ 416 (5) in 50 mM-Tes/50 mM-NaCl/15 mM-MgCl₂/0.01% gelatin, pH 7.0, by the method of Halford *et al.* (1980); from each reaction mixture of 300 μ l, 13 samples of 20 μ l were withdrawn at timed intervals after the start of the reaction, the reaction was terminated immediately within each sample, and the amount of product present within each timed sample was determined after electrophoresis through an agarose gel.

A further comparison between equilibrium and kinetic experiments was made by attempting to saturate samples of λ 416 (5) DNA with increasing concentrations of the *Eco*RI enzyme in two series of experiments (Fig. 3). In the first series, a fixed concentration of λ 416 (5) DNA was mixed with a range of *Eco*RI enzyme concentrations in the absence of MgCl₂, and the formation of the equilibrium complex was measured by the filter binding technique; this titration produced a hyperbolic equilibrium binding curve (Fig. 3), and a value for K_D of 0.4 nM. In the second series, a fixed concentration of λ 416 (5) DNA was mixed with a range of *Eco*RI enzyme concentrations in the presence of MgCl₂, and in each of these mixtures the first order rate constants (k_b) that describes the formation of the final DNA products was measured. At the highest enzyme concentrations used in Fig. 3, samples for product analysis were withdrawn from the reaction mixtures at 10 s intervals as the reaction reached completion in about 60 s. Consequently, the series of kinetic experiments could not be extended to higher enzyme concentrations, as the reactions were then too fast for the available technique. But over the range of enzyme concentrations tested, the values of k_b were found to be linearly dependent upon the concentration of the *Eco*RI enzyme (Fig. 3). Hence the highest enzyme concentrations used in this series must be considerably below the value of K_m^* , where K_m^* is equivalent to the enzyme concentration at which the apparent rate constant from a single turnover reaction is one half of its maximal value: thus $K_m^* \gg 4$ nM. [In the kinetics of a single turnover reaction, the first-order rate constant describing product formation, where no distinction is made between enzyme-bound and free product, should follow a hyperbolic relationship against the enzyme concentration which may be defined by the parameter K_m^* ; see eqn. (7) of Halford *et al.* (1980).]

The two series of experiments in Fig. 3 indicate that the apparent affinity of the *Eco*RI restriction enzyme for its recognition site on λ 416 (5) DNA is at least 10-fold higher when measured by the K_D value obtained in equilibrium binding experiments than it is from the K_m^* value obtained in kinetic experiments. For the minimal enzyme mechanism:

it follows that $K_D = k_{-1}/k_1$ whereas $K_m^* = (k_{-1} + k_2)/k_1$, and thus one possible explanation for the difference between K_D and K_m^* is that the value of the rate constant, k_2 , is more than 10-fold higher than the value for k_{-1} . But the variations in $k_{\text{cat.}}/K_m$ of the *Eco*RI restriction enzyme towards λ 416 (5) DNA, that are caused by alterations to either pH or ionic strength, are readily accounted for by changes

in the ratio of k_2/k_{-1} from above to below 1 (Halford *et al.*, 1980); hence we conclude that the ratio of k_2/k_{-1} will not be greater than 10 under these experimental conditions. However, the experimental conditions used in the filter binding experiments in Fig. 3 were not identical with those used in the kinetic experiments. Gelatin was present in the kinetic experiments, but, as gelatin affects the stability, and not the activity, of the *EcoRI* enzyme (Greene *et al.*, 1974), we do not consider this difference to be significant. In addition, the solutions for the kinetic experiments, but not the equilibrium studies, contained MgCl_2 . Therefore one interpretation of the data in Fig. 3 is that the equilibrium dissociation constant of the *EcoRI* restriction endonuclease from its recognition site on DNA is increased by the presence of Mg^{2+} .

Binding at sequences different from the *EcoRI* recognition site

We have also carried out equilibrium binding studies between the *EcoRI* restriction endonuclease

and the DNA from $\lambda 395$ (0), as this DNA lacks *EcoRI* recognition sites and thus the binding of the enzyme must occur at non-specific nucleotide sequences. Moreover, in contrast with DNA molecules that contain the *EcoRI* recognition site, the effect of Mg^{2+} upon the binding of the enzyme to $\lambda 395$ (0) DNA could also be examined. The binding of the *EcoRI* enzyme to $\lambda 395$ (0) DNA was studied in a series of filter binding experiments in which a fixed concentration of the DNA was first titrated with the enzyme in the absence of MgCl_2 ; this series was then repeated under identical conditions except for the addition of selected MgCl_2 concentrations (Fig. 4).

As had been noticed previously (under slightly different conditions; Fig. 1), the interaction between the *EcoRI* restriction enzyme and $\lambda 395$ (0) DNA, in the absence of Mg^{2+} , followed a sigmoidal equilibrium binding curve and analysis of the data in Fig. 4 by using eqn. (2) yielded a non-linear Eadie plot with the same direction of curvature as in Fig. 1(b). In the presence of Mg^{2+} , the equilibrium binding curves retained the sigmoidal character and again gave rise to curved Eadie plots, but the affinity of the enzyme for $\lambda 395$ (0) DNA appeared to be progressively decreased by the addition of MgCl_2 . For the experiments in Fig. 4, the concentration of the *EcoRI* enzyme that was required to retain 50% of the $\lambda 395$ (0) DNA on the filter rose from 2.1 nM without MgCl_2 to 3.8 nM with 5 mM- MgCl_2 and to 6 nM with 15 mM- MgCl_2 .

The simplest mechanism with which we have been able to accommodate both the sigmoidicity and the effect of Mg^{2+} upon these binding curves is:

where M, E and D are Mg^{2+} ions, the *EcoRI* enzyme and $\lambda 395$ (0) DNA respectively, EM is a complex between the enzyme and Mg^{2+} that cannot bind to DNA, ED and E_2D are complexes between the enzyme and one molecule of DNA that contain one and two molecules of enzyme respectively. Eqn. (4) is defined by three equilibrium dissociation constants:

$$K_1 = \frac{[\text{E}][\text{D}]}{[\text{ED}]} \quad (5)$$

$$K_2 = \frac{[\text{E}][\text{ED}]}{[\text{E}_2\text{D}]} \quad (6)$$

$$K_3 = \frac{[\text{E}][\text{M}]}{[\text{EM}]} \quad (7)$$

Fig. 4. Binding to DNA without recognition sites. Samples containing the concentration of *EcoRI* restriction endonuclease indicated by $[\text{E}_0]$ and 30 μM - $[\text{H}^3]$ DNA from $\lambda 395$ (0) in 50 mM-Tes/50 mM-NaCl, pH 7.0 and one of the following concentrations of MgCl_2 were individually drawn through a nitrocellulose membrane filter and the radioactivity retained on each filter was measured: O, zero MgCl_2 ; ●, 5 mM- MgCl_2 ; □, 15 mM- MgCl_2 . For the titration at each MgCl_2 concentration, additional samples containing concentrations of *EcoRI* restriction endonuclease in the range 15–85 nM were filtered as above, and from these samples the radioactivity retained on the filter in the presence of an infinite concentration of enzyme was determined by a double reciprocal plot; the percentage saturation of the DNA at each enzyme concentration was then evaluated from the proportion of the maximal retention of radioactivity.

where each concentration term is the equilibrium concentration; total concentrations are denoted by $[M_0]$, $[E_0]$ and $[D_0]$. But under the experimental conditions where $[M_0] \gg [E_0] \gg [D_0]$, the following relationships hold between total and equilibrium concentrations:

$$[M_0] = [M] \quad (8)$$

$$[E_0] = [E] \cdot \left(1 + \frac{[M_0]}{K_3} \right) \quad (9)$$

(At the concentrations of $MgCl_2$ and DNA used here, even the binding of one Mg^{2+} ion to every phosphate group on the DNA would not significantly affect the free Mg^{2+} concentration.) We also utilize the observed values for an apparent equilibrium dissociation constant, K_0 , for the binding of the enzyme to this DNA where K_0 is defined by:

$$K_0 = \frac{[E_0][D]}{[ED + E_2D]} \quad (10)$$

At each enzyme concentration used for the experiments in Fig. 4, a value for K_0 may be obtained if it is assumed that the radioactivity retained on the filter is proportional to the concentrations of all enzyme-DNA complexes ($[ED] + [E_2D]$), and hence subtraction of this value from $[D_0]$ yields the free DNA concentration, $[D]$. Substitution from eqns. (5), (6), (8) and (9) into eqn. (10) yields the relationship:

$$\frac{1}{K_0} = \frac{1}{K_1 \cdot \left(1 + \frac{[M_0]}{K_3} \right)} + \frac{[E_0]}{K_1 \cdot K_2 \cdot \left(1 + \frac{[M_0]}{K_3} \right)^2} \quad (11)$$

Thus the mechanism in eqn. (4) demands a linear relationship between $1/K_0$ and $[E_0]$ at any single Mg^{2+} concentration (including zero Mg^{2+}) but both the ordinate intercept and the gradient of such a plot must vary with the Mg^{2+} concentration.

The data in Fig. 4 for the binding of the *EcoRI* restriction endonuclease to λ 395 (0) DNA were analysed with eqn. (11) (Fig. 5). In the absence of $MgCl_2$, a linear relationship was noted between the values of $1/K_0$ and the total enzyme concentrations, $[E_0]$; the gradient and the intercept of this plot yielded values for K_1 at 6 nM and K_2 at 1.5 nM. (The deviation from linearity at the end of the plot in Fig. 5 is not significant because accurate values for K_0 cannot be determined at high enzyme concentrations where more than 90% of the DNA is retained on the filter.) In the presence of 15 mM- $MgCl_2$, $1/K_0$ was again found to be linearly related

Fig. 5. Analysis of sigmoidal binding curves. A value of K_0 was determined by application of eqn. (10) to each point in the filter binding curves shown in Fig. 4, at varied concentrations of the *EcoRI* restriction endonuclease and 30 pM-DNA from λ 395 (0) in 50 mM-Tes/50 mM-NaCl, pH 7.0 and either zero $MgCl_2$ (O) or 15 mM- $MgCl_2$ (□); the values for $1/K_0$ obtained at each concentration of the *EcoRI* restriction endonuclease, $[E_0]$, were then plotted against $[E_0]$.

to $[E_0]$, and from both the gradient and the intercept of this plot, together with the values of K_1 and K_2 determined in the absence of $MgCl_2$, K_3 was evaluated at 7 ± 3 nM. The same value for K_3 was also found by a similar analysis, not shown here, of the data in Fig. 4, obtained at 5 mM- $MgCl_2$. For the same binding reaction in the absence of $MgCl_2$, but at 100 mM-NaCl (Fig. 1), a linear relationship was again obtained between $1/K_0$ and $[E_0]$, from which K_1 and K_2 were determined at 6 nM and 0.7 nM respectively. Thus, the interactions between the *EcoRI* restriction enzyme and λ 395 (0) DNA are fully compatible with the binding mechanism in eqn. (4). An alternative mechanism, where Mg^{2+} inhibits the binding of the enzyme to DNA by forming a Mg^{2+} -DNA complex which cannot bind to the enzyme, can be shown to be incompatible with the experimental data.

We now consider the two complexes formed between the *EcoRI* enzyme and this DNA. If, in the absence of recognition sites, the enzyme binds to any DNA sequence, then there exist a large number of potential binding sites on λ 395 (0) DNA. But the two equilibrium dissociation constants (eqns. 5 and 6) cannot refer to two independent sites on the DNA, for if the separate binding of the protein to either one of these sites was sufficient to retain the DNA on the filter, then the filter binding studies would have

produced a hyperbolic binding curve that measured only the binding of the enzyme to the site for which it has the highest affinity. Yet other proteins have been found to yield sigmoidal binding curves to DNA in filter binding assays. The sigmoidal binding curve between the λ repressor and its operator has been assigned to the assembly of an active repressor protein from inactive subunits (Ptashne, 1971), but the *EcoRI* restriction enzyme exists in its active dimeric structure at the protein concentrations used here (Modrich & Zabel, 1976). Likewise, the helix-destabilizing protein from bacteriophage T4 binds co-operatively to DNA, which may be due to either the binding of one protein molecule denaturing a segment of duplex DNA or to interactions between protein molecules on the DNA (Alberts & Frey, 1970). But the *EcoRI* restriction enzyme displays similar reactivities to both the supercoiled and linear forms of plasmid pMB9 (Halford *et al.*, 1979) so its binding to DNA is unlikely to alter the helical structure of the DNA. Therefore, we suggest the following structures for the two complexes between the *EcoRI* enzyme and the DNA from λ 395 (0) in eqn. (4); first the enzyme binds, with an equilibrium constant K_1 , to any location along this DNA and then this complex binds, with an equilibrium constant K_2 , a second molecule of enzyme with the binding of the second enzyme taking place onto the protein already attached to the DNA. It is known that the *EcoRI* restriction enzyme will aggregate from its dimeric subunit structure to a tetramer at high protein concentration (Modrich & Zabel, 1976) and it is possible that the equilibrium dissociation constant between the tetrameric and dimeric forms of the enzyme is smaller for enzyme bound to non-specific DNA than for enzyme free in solution. Alterations to the subunit assembly of proteins frequently accompany their interactions with ligands (Frieden, 1971).

However, the observation that co-operative binding is only observed with DNA molecules that lack *EcoRI* recognition sites such as that from λ 395 (0), and not with DNA molecules that contain *EcoRI* sites such as those from λ 401 (2) or λ 416 (5), implies that the conformation of the *EcoRI* restriction endonuclease bound to non-specific DNA must differ from the enzyme conformations both when bound to DNA at an *EcoRI* recognition site and also when free in solution.

Specificity of EcoRI

In 50 mM-NaCl, pH 7.0, the *EcoRI* restriction endonuclease binds to its recognition site on DNA with a equilibrium dissociation constant of 0.5 nM; this value is the average of the K_D determinations with the DNA from λ 401 (2) and λ 416 (5), which both contain a single *EcoRI* recognition site (Fig. 2).

But, under the same conditions, the enzyme binds to DNA lacking *EcoRI* recognition sites, that from λ 395 (0), by a more complicated mechanism in which the initial interaction between the enzyme and this DNA features an equilibrium dissociation constant, K_1 , of 6 nM (Fig. 5). Thus the affinity of the *EcoRI* restriction enzyme for either λ 401 (2) or λ 416 (5) DNA appears to be about 10-fold higher than for λ 395 (0) DNA. Although the complex formed with either λ 401 (2) or λ 416 (5) DNA must be at the *EcoRI* recognition site, the binding of the enzyme to λ 395 (0) DNA can presumably occur at any location on this DNA. The DNA contains about 4×10^4 base pairs (Davidson & Szybalski, 1971), and, if we assume that every base pair constitutes the start of a non-specific binding site, then the equilibrium dissociation constant for the binding of the *EcoRI* restriction enzyme to its recognition site on DNA is smaller by a factor of about 4×10^5 than that for any individual site on DNA apart from the recognition sequence. The question now is whether this factor between the specific and non-specific binding equilibria is sufficient to account for the specificity that the *EcoRI* restriction enzyme displays in the hydrolysis of DNA.

We have measured approximately the catalytic specificity of the *EcoRI* restriction endonuclease by comparing its ability to cleave two circular DNA duplexes of similar molecular weight, plasmid pMB9 and the DNA (replicative form) from bacteriophage ϕ X174 (Fig. 6). Circular DNA molecules were used because the cleavage of one phosphodiester bond on circular DNA can alter its electrophoretic mobility (Johnson & Grossman, 1977). The plasmid pMB9, when isolated from a *recA* strain of *Escherichia coli* in its monomeric form, contains a single *EcoRI* recognition site and may be prepared as more than 95% supercoiled DNA (Halford *et al.*, 1979). The *EcoRI* restriction enzyme first cleaves one strand of supercoiled pMB9 at the recognition site to generate the open-circle form of this DNA and then, in a separate reaction, cleaves the second strand of the recognition site to produce the linear DNA. Under the experimental conditions in Fig. 6, the supercoiled form of plasmid pMB9 was eliminated by digesting the DNA with between 0.25 and 2.5 units of enzyme activity. In contrast, the DNA from ϕ X174 (replicative form) does not possess an *EcoRI* recognition site among its 5386 base pairs (Sanger *et al.*, 1978); the preparation of this DNA used here was a mixture of the supercoiled and open-circle forms of the DNA, the latter generated spontaneously by prolonged storage, with a small portion of the linear form also present. The *EcoRI* restriction enzyme was able to convert the supercoiled form of ϕ X174 DNA into open circles but only at much higher enzyme concentrations than those required for the reaction

Fig. 6. *Cleavage of DNA by EcoRI*

Reaction mixtures (20 μ l) containing 0.35 μ g of DNA from either plasmid pMB9 or phage ϕ X174 (replicative form), as indicated above, in 100 mM-Tris/100 mM-NaCl/10 mM-MgCl₂, pH 7.5, were incubated for 30 min at 37°C in the presence of 1 μ l of *EcoRI* restriction endonuclease at one of the following enzyme concentrations [units of enzyme activity, as defined by Halford *et al.* (1979), per μ l]: slots A, zero units; slots B, 0.25 units; slots C, 2.5 units; slots D, 25 units; slots E, 250 units; slots F, 2500 units; slots G, 25000 units. After the incubation, 10 μ l of 100 mM-EDTA/10% (w/v) Ficoll 400/0.05% Bromophenol Blue, pH 8.0, was added to each reaction mixture, each sample was loaded into one slot of an agarose-slab gel and then subjected to electrophoresis as described by Halford *et al.* (1979). The mobilities of the supercoiled, open-circle and linear forms of these DNA molecules are marked on the gel.

on plasmid pMB9; the elimination of the supercoiled ϕ X174 DNA was achieved by 2.5×10^3 – 2.5×10^4 units of enzyme activity among the digests in Fig. 6. Moreover, none of the linear form of ϕ X174 DNA was produced by even the highest concentration of the *EcoRI* enzyme used here; if the single-strand scissions that generate the open-circles are at random locations on this DNA, it will be statistically improbable for linear DNA to be obtained by two single strand scissions at similar locations but on opposite strands of the duplex.

Thus the amounts of the *EcoRI* restriction endonuclease that are required to cleave one strand of duplex DNA on plasmid pMB9 and on ϕ X174 DNA differ by a factor of about 1×10^4 . But whereas the reaction on pMB9 occurs at its single *EcoRI* recognition site, the reaction on ϕ X174 DNA can presumably occur between any base pair on this DNA. Hence the reactivity of the *EcoRI* restriction enzyme towards its recognition site on DNA is larger by a factor of about 5×10^7 than that towards

an individual site among other DNA sequences. This factor for the catalytic specificity of the *EcoRI* restriction endonucleases is considerably larger than those that have been obtained for other enzymes, such as chymotrypsin, by comparing k_{cat}/K_m values for their true substrates against substrate analogues (Fersht, 1977). Even so, it is likely to be an underestimate; firstly, the cleavage of ϕ X174 DNA may be due to a non-specific nuclease that is present as a trace contaminant in our preparations of the *EcoRI* restriction enzyme; secondly, the DNA from ϕ X174 contains numerous copies of nucleotide sequences that differ by one or two base pairs from the *EcoRI* recognition sequence and, if the reaction is occurring at such sequences, then the true reaction rate at a random DNA sequence will be even lower than the rate observed on ϕ X174 DNA.

The discrimination shown by the *EcoRI* restriction endonuclease between its recognition site and each alternative sequence of bases on DNA yields a factor of 4×10^5 when measured from equilibrium binding studies and a factor of greater than 5×10^7 when measured from the catalytic reaction. Thus, the different binding affinities of the enzyme between its recognition site and other sequences make a major contribution to the specificity of this enzyme for preferential cleavages of DNA at the recognition site, but the catalytic specificity cannot be accounted for solely by differences in binding energies. However, our filter binding experiments have indicated that the conformation of the *EcoRI* restriction enzyme, when bound to the recognition site on DNA, differs from that when bound to other DNA sequences. Therefore we suggest that the conformation of the enzyme bound to the recognition site is an active conformation that can catalyse the hydrolysis of the DNA, whereas the conformation of the enzyme bound to other DNA sequences is catalytically inactive. We have also suggested that the binding of the *EcoRI* enzyme to DNA sequences that differ from the *EcoRI* recognition site may be accompanied by the tetramerization of the enzyme; on this hypothesis, the tetramer would function as a 'safety device' by stabilizing the inactive conformation of the enzyme whenever it binds to non-specific sequences on the DNA.

Catalysis by EcoRI

The filter binding experiments with the *EcoRI* enzyme and λ DNA molecules that contain *EcoRI* recognition sites were carried out in the absence of Mg²⁺. One of many possible hypotheses from the work of Goppelt *et al.* (1980) is that Mg²⁺ enhances the affinity of the enzyme for synthetic oligonucleotides. But two experiments in the present paper suggest that Mg²⁺ causes a major decrease in the stability of the complex between the *EcoRI* enzyme and its recognition site on native DNA.

Firstly, with the DNA from λ 416 (5) that has an *EcoRI* site, one interpretation of the data in Fig. 3 is that K_m^* (from the rates of DNA cleavage) is greater than K_D (from equilibrium binding) because only the former was measured in the presence of Mg^{2+} . Secondly, with the DNA from λ 395 (0) that lacks *EcoRI* sites, the data in Fig. 4 show directly that Mg^{2+} decreases the affinity of the enzyme for this DNA. But eqn. (4), where the enzyme- Mg^{2+} complex cannot bind to λ 395 (0) DNA, is likely to be an over-simplification of the mechanism by which the *EcoRI* restriction enzyme binds, in the presence of Mg^{2+} , to DNA containing a recognition site. The majority of Mg^{2+} -requiring enzymes that catalyse reactions on phosphate esters form an active complex containing the metal ion (Mildvan, 1970) and thus it is reasonable to suggest that the cleavage of DNA by the *EcoRI* restriction enzyme requires the formation of a ternary complex, EMS, with both Mg^{2+} and the recognition sequence bound simultaneously to the enzyme:

where E, M and S represent the *EcoRI* enzyme, Mg^{2+} ions and the recognition site on DNA respectively. But, given the decrease in the binding of the enzyme to DNA in the presence of Mg^{2+} , the ternary complex of EMS must be unstable relative to the binary complexes containing either enzyme and Mg^{2+} (EM) or enzyme and DNA (ES); i.e. the equilibrium dissociation constant of the DNA from the EM complex is much larger than that from the free enzyme. The relative instability of the ternary EMS complex would explain the observation of Roulland—Dussoix *et al.* (1974) that the activity of the *EcoRI* restriction enzyme increases with increasing $MgCl_2$ concentration up to about 15 mM, but then decreases at higher $MgCl_2$ concentrations. Other restriction endonucleases, apart from *EcoRI*, are also inhibited by high concentrations of $MgCl_2$ (Clarke & Hartley, 1979).

Therefore, it appears that Mg^{2+} plays two roles in the reaction catalysed by the *EcoRI* restriction endonuclease. First, it is likely to be involved in the hydrolytic reaction as the DNA is cleaved. Secondly, Mg^{2+} causes the destabilization of the complex between the enzyme and DNA so that the free energy change for the formation of the EMS complex is less negative than that for the formation of the ES complex. Hence the interaction between Mg^{2+} and the enzyme-DNA complex decreases the activation energy barrier between the enzyme-bound substrate and reaction products and thus accelerates the rate of this step within the reaction mechanism. In this role, Mg^{2+} is effectively liberating the binding energy of the enzyme-DNA com-

plex for use in the catalytic reaction. The destabilization of the enzyme-DNA complex by Mg^{2+} is not the only device that the *EcoRI* restriction endonuclease is considered to employ to minimize the activation energy barrier between enzyme-bound substrate and products, for Halford *et al.* (1980) suggested that supplementary interactions were formed between the *EcoRI* enzyme and nucleotides adjacent to the *EcoRI* recognition site only at the transition state of the DNA cleavage reaction and not upon the formation of the initial complex of the enzyme at the recognition site; supplementary interactions formed at this stage of the reaction would decrease the activation energy barrier by stabilizing the transition state relative to the ground state. The inter-relationships between enzyme specificity and catalysis, and substrate binding energies, have been discussed by Jencks (1975) and the *EcoRI* restriction endonuclease provides an experimental system in which the general theory of Jencks (1975) appears to be valid.

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