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The *Eco*RI restriction endonuclease with bacteriophage λ DNA

Kinetic studies

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The kinetics of the reactions of the *Eco*RI restriction endonuclease at individual recognition sites on the DNA from bacteriophage λ were found to differ markedly from site to site. Under certain conditions of pH and ionic strength, the rates for the cleavage of the DNA were the same at each recognition site. But under altered experimental conditions, different reaction rates were observed at each recognition site. These results are consistent with a mechanism in which the kinetic stability of the complex between the enzyme and the recognition site on the DNA differs among the sites, due to the effect of interactions between the enzyme and DNA sequences surrounding each recognition site upon the transition state of the reaction. Reactions at individual sites on a DNA molecule containing more than one recognition site were found to be independent of each other, thus excluding the possibility of a processive mechanism for the *Eco*RI enzyme. The consequences of these observations are discussed with regard to both DNA–protein interactions and to the application of restriction enzymes in the study of the structure of DNA molecules.

The *Eco*RI restriction endonuclease (EC 3.1.23.13) cleaves both strands of double-stranded DNA at the recognition sequence:

$$\begin{array}{l} 5'\text{-G}\downarrow\text{A-A-T-T-C-}3' \\ 3'\text{-C-T-T-A-A-G}\uparrow\text{-}5' \end{array}$$

where the arrows indicate the bonds that are cleaved; Mg^{2+} is the only cofactor required for this reaction (Hedgpeth *et al.*, 1972). The overall process, though not the chemical mechanism of catalysis, by which the *Eco*RI restriction enzyme cleaves both strands of the DNA is known; this enzyme cleaves the susceptible phosphodiester bond on only one strand of the DNA at a time and thus requires two separate reactions to complete the double strand scission (Halford *et al.*, 1979). The experiments described in this and the accompanying paper (Halford & Johnson, 1980) are now concerned with the mechanism by which this enzyme achieves its specificity.

The *Eco*RI restriction endonuclease will cleave double-stranded DNA wherever its recognition site occurs on the DNA, there being no limitation upon the base pairs immediately adjacent to the hexanucleotide recognition site (Garfin *et al.*, 1975). However, different recognition sites on the same DNA molecules may be cleaved by this enzyme at different rates (Thomas & Davis, 1975; Forsblom *et al.*, 1976). For example, the DNA from bacteriophage λ contains five *Eco*RI recognition sites numbered *sr*I 1 to *sr*I 5 from left to right on the linear map of λ DNA (see Fig. 1 below). Thomas & Davis (1975) examined under the electron microscope molecules of λ DNA that had been cleaved only once by this enzyme: the largest portion of DNA molecules were found to receive their first cut at *sr*I 5, the second largest at *sr*I 4 and progressively fewer at each of the remaining sites.

In the present study, we have extended the observations of Thomas & Davis (1975) by examining the reaction of the *Eco*RI restriction enzyme at the separate recognition sites on λ DNA under a variety of experimental conditions to identify the factors that cause different recognition sites to be cleaved at different rates. As the DNA from wild-type bacteriophage λ contains five *Eco*RI recognition sites, this could not be used as a substrate to obtain accurate kinetic data about the reactions at individual sites. Consequently, we have

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

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used in most of these experiments a series of DNA substrates obtained from derivatives of bacteriophage λ, each of which contain a single *Eco*RI recognition site with the other four being eliminated by mutations (Murray & Murray, 1974).

Materials and methods

Enzymes

The nomenclature of Smith & Nathans (1973) was employed for restriction endonucleases and their recognition sites except that the prefix endo-R has been omitted.

The *Eco*RI restriction endonuclease was purified from *Escherichia coli* RY13 by the method of A. Atkinson & A. Bingham (C.A.M.R., Porton, Salisbury, Wilts., U.K.) as described previously (Halford *et al.*, 1979). The enzyme was stored at −20°C in 10mM-K_2HPO_4/200mM-NaCl/10mM-β-mercaptoethanol/0.2% Triton X-100/50% (w/v) glycerol, pH 7.5, where no loss of activity was noted over a 1 year period. Dilutions of the enzyme were made in the same buffer also containing 0.1 mg of autoclaved gelatin/ml (Greene *et al.*, 1974). The protein concentration of stock solutions was determined by the recovery of amino acids after hydrolysis in 6M-HCl at 105°C (Williams, 1974).

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Williams, 1974) revealed that about 90% of the protein in the stock solutions was a single polypeptide with mol.wt. 29000. Both this subunit molecular weight and the amino acid composition agreed closely with those reported for the *Eco*RI restriction enzyme (Modrich & Zabel, 1976). Since the enzyme is a dimer in solution (Roulland-Dussoix *et al.*, 1974), the molarities of *Eco*RI enzyme solutions given here are for a mol.wt. of 58000. The enzyme can aggregate to a tetramer at high concentrations (Modrich & Zabel, 1976), but very little tetramer would have been formed at the highest enzyme concentrations employed here (0.01μM). The molarities of the dimeric enzyme have not been corrected for the loss of amino acids during determination of the protein concentration, or for the heterogeneity of the preparations, and it has also been assumed that all the protein is active enzyme. [Concentrations of restriction enzyme solutions are often given in terms of units of enzyme activity; with the units defined by Halford *et al.* (1979), a solution of 1.0nM-*Eco*RI restriction enzyme corresponds to approx. 500units/ml.]

The *Sal*I restriction endonuclease (EC 3.1.23.37; Arrand *et al.*, 1978) was partially purified from *Streptomyces albus* G by the method of Greene *et al.* (1978) followed by chromatography on Sephadex A-50 (A. Maxwell & S. E. Halford, unpublished work). We had noted previously that the *Sal*I restriction enzyme inhibited the *Eco*RI enzyme and had suggested that this effect might be due to impurities in the preparations of *Sal*I used at that time that were binding to the DNA (Halford *et al.*, 1979). With the purer preparations of the *Sal*I enzyme used here, no difference could be detected between the rates of *Eco*RI reactions in the presence or absence of *Sal*I.

DNA

The following derivatives of bacteriophage λ were used: wild type λ^+; λ395 with mutations *b*538 (∇*sr*I 1-2), *sr*I 3°4°5°; λ401 with mutations *b*519 (∇*sr*I 1), *sr*I 3°4°5°; λ416 with mutations *b*538 (∇*sr*I 1-2), *sr*I 3°4°, *cI*857, S *am* 7; λ421 with mutations *b*538 (∇*sr*I 1-2), *sr*I 3°5°, *cI*857; λSH1 with mutations *b*538 (∇*sr*I 1-2), *sr*I 3°. The phage λ^+ was from this laboratory, λSH1 was constructed as below and all other phages were from Professor K. Murray (Department of Molecular Biology, University of Edinburgh, Edinburgh, U.K.); these are described by Murray & Murray (1974). The mutant phage will be denoted here as λ395 (0), λ401 (2), λ416 (5), λ421 (4) and λSH1 (4,5) where the numbers in brackets specify each recognition site out of the five *Eco*RI sites on λ^+ DNA that are still present except that the DNA from λ395 (0) contains none of the sites. Maps of the DNA from these phages are shown in Fig. 1. The preparation of phage and the purification of phage DNA were as described previously (Halford *et al.*, 1979) except that λ416 (5) was grown on *E. coli* Y MEL *Su* III (from Professor Murray) instead of *E. coli* C600 *thy*$^-$ and that for all preparations involving [*methyl*-^{3}H]thymidine, 2-deoxyadenosine (250μg/ml) was added at the same time as the phage and the [^{3}H]thymidine. The DNA solutions were stored at 4°C in 10mM-Tris/HCl/0.1mM-EDTA, pH 7.5, over chloroform.

The concentrations of all DNA solutions were determined from their absorbance by using the value $A_{260}^{0.1\%} = 20$. The molecular weight of the DNA from λ^+ was taken as 30.8×10^6 and that for the DNA of the mutant phages was calculated from this figure given the sizes of the deletion mutations (Davidson & Szybalski, 1971). The molarities of DNA solutions are given in terms of DNA molecules and not base pairs.

The phage λSH1 (4,5) was constructed by digesting 4μg of each of the DNA from λ^+ and λ395 (0) with 20 units of *Sal*I restriction endonuclease in 50mM-Tris/HCl/100mM-NaCl/5mM-$MgCl_2$, pH 7.5 for 1h at 37°C. The solution was then heated to 67°C for 1h and later treated with DNA ligase for 3h at 10°C as described by Hepburn & Hindley (1979). This DNA, after precipitation with sodium acetate and isopropanol and resuspension in 10mM-Tris/HCl/1.0mM-EDTA, pH 7.5, was used to transfect *E. coli* C600 and individual plaques were devel-

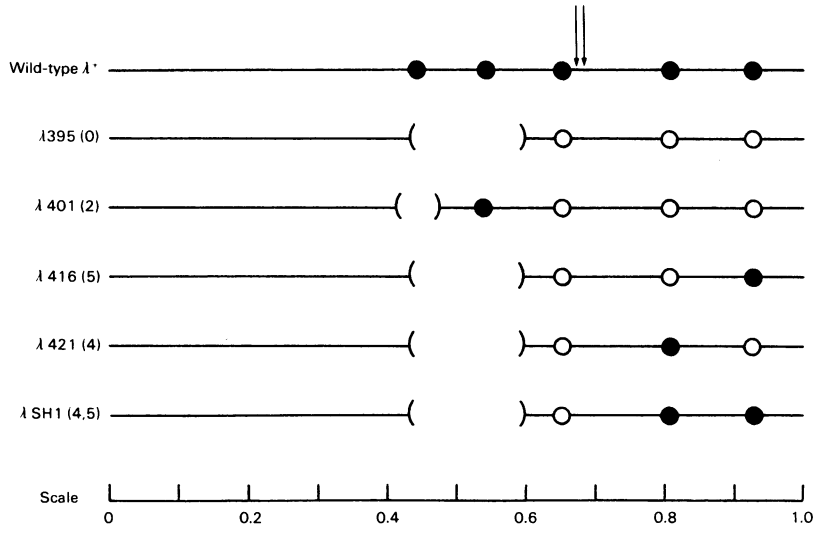

Fig. 1. *Maps of DNA from bacteriophage λ derivatives*

The maps of the DNA from bacteriophage λ^+ and the mutant bacteriophages used here are shown: ●, *Eco*RI recognition site (Thomas & Davis, 1975); ○, *Eco*RI recognition site removed by mutation (Murray & Murray, 1974): ↓, *Sal*I recognition site (Arrand *et al.*, 1978); ( ), deletion mutation (Davidson & Szybalski, 1971). The map co-ordinates are defined on the scale from 0.0 at *cos* L to 1.0 at *cos* R. The *Sal*I recognition sites marked on λ^+ DNA are also present on all the derivatives, but, in the case of λSH1 (4,5) DNA, the orientation of the DNA fragment between the two *Sal*I sites is not known.

oped from the transfectants (Hohn & Murray, 1977). A number of these plaques were excised from the agar and each was resuspended in 2.0ml of *E. coli* C600 (growing exponentially) in nutrient broth containing 10mM-$MgCl_2$. These suspension were shaken overnight at 37°C. The lysed cultures were centrifuged in a benchtop centrifuge and 0.7ml samples of the supernatants were treated with 10μg each of pancreatic DNAase and RNAase for 30min at 37°C. The solutions were then adjusted to 50mM-EDTA, washed once with phenol and twice with chloroform, and the DNA was precipitated as before. The DNA was resuspended in 20μl of 10mM-Tris/HCl/0.1mM-EDTA, pH7.5, and this was used for restriction enzyme digests (Halford *et al.*, 1979). One plaque was found, by digestion with the *Eco*RI, *Sal*I and *Hin*dIII enzymes, to contain the requisite DNA, featuring only *sr*I 4 and *sr*I 5 (originally present on λ^+) and both the *b*538 deletion (▽*sr*I 1-2) and *sr*I 3° [originally present on λ395 (0)] and this phage was designated λSH1.

Kinetic methods

The cleavage of both strands of the DNA from λ416 (5), of mol.wt. 25.8×10^6, by the *Eco*RI restriction endonuclease yields two products of mol.wts. 23.7×10^6 and 2.13×10^6. The rate of this reaction can be monitored by removing samples at various times from the reaction mixture, immediately terminating the reaction in each sample and then separating the low-molecular-weight product from the substrate by agarose-gel electrophoresis. The reaction of the *Eco*RI enzyme on the DNA from λ421 (4) can be monitored similarly by the appearance of a low-molecular weight product (Halford *et al.*, 1979). But the DNA from λ401 (2), of mol.wt. 28.9×10^6, is cleaved by *Eco*RI into two products of mol.wt. 14.8×10^6 and 14.1×10^6, neither of which can be separated readily from the substrate. However a double digest of this DNA with both the *Eco*RI and *Sal*I restriction enzymes yields four products of mol.wts. 14.8×10^6, 9.76×10^6, 4.00×10^6 and 0.31×10^6; insufficient DNA is used in our experiments to detect the smallest product. Thus by completely digesting λ401 (2) DNA with *Sal*I, the rate of the *Eco*RI-catalysed reaction can be measured from the appearance of the product of mol.wt. 4.0×10^6; this DNA is that between the single *Eco*RI and the left-hand *Sal*I recognition site on λ401 (2) DNA (Fig. 1).

A typical kinetic experiment is described (Fig. 2); 4.0μg of ^{3}H-labelled DNA from λ401 (2) in 10mM-Tris/HCl/0.1mM-EDTA, pH7.5, was first digested overnight at 37°C with 50 units of *Sal*I restriction endonuclease in the presence of the amounts of NaCl and $MgCl_2$ required for the subsequent *Eco*RI reaction. After heating to 67°C to inactivate the *Sal*I enzyme and quenching on ice,

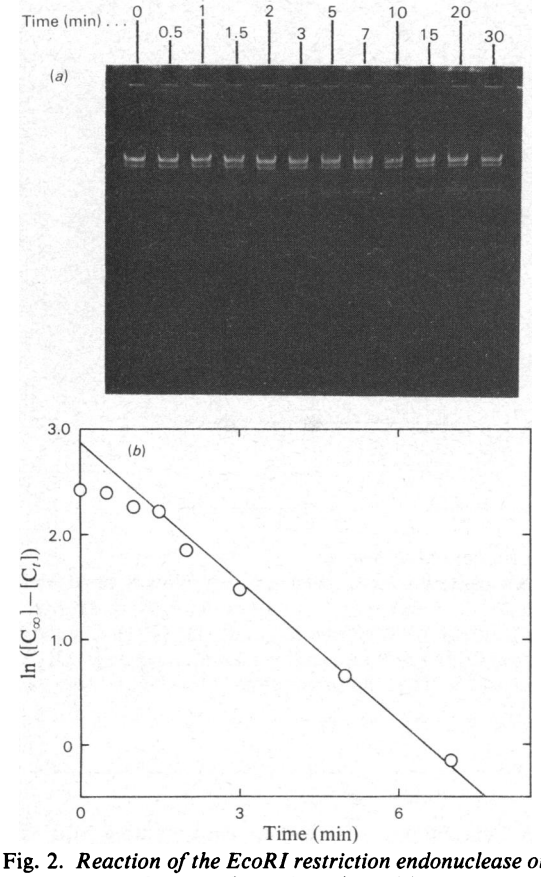

Fig. 2. *Reaction of the EcoRI restriction endonuclease on the DNA from phage λ401 (2)*

(*a*) Analysis by electrophoresis through a 1.0% (w/v) agarose gel of samples, withdrawn at the times (in min) specified by each slot on the gel, from a reaction mixture containing 0.48 nM-*Eco*RI restriction endonuclease and 0.5 nM-DNA (^{3}H-labelled) from phage λ401 (2) in 50 mM-Tes/50 mM-NaCl/15 mM-$MgCl_2$/0.01% gelatin, pH 7.0, at 22°C. The DNA from λ401 (2) had been cleaved with the *Sal*I restriction enzyme before the above reaction. (*b*) Semi-logarithmic plot determination of the rate constant, k_b, for the formation of product in the reaction in (*a*); $[C_t]$ is measured from the radioactivity in the slice of the above gel containing the DNA product of mol.wt. 4.0×10^6 at time t and $[C_\infty]$ is the average from all samples taken after the reaction had reached completion: an 'infinity' time point was not taken in this particular experiment. The line drawn is that used in the evaluation of k_b.

further components were added to the reaction mixture to produce a solution (300 μl) containing 0.5 nM-[^{3}H]DNA from λ401 (2), already cleaved at the *Sal*I sites, in 50 mM-Tes/50 mM-NaCl/15 mM-$MgCl_2$/0.01% gelatin, pH 7.0. Upon incubation at 22°C, the *Eco*RI reaction was initiated by the addition of 5 μl of *Eco*RI restriction endonuclease (75 units), yielding a final *Eco*RI concentration of 0.48 nM. At timed intervals after the addition of the *Eco*RI enzyme, samples (20 μl) were removed and immediately mixed with 10 μl of stop solution (10% Ficoll 400/0.1 M-EDTA/0.05% Bromophenol Blue, pH 8.0); the sample for the zero-time point was removed before the addition of *Eco*RI and that for the infinity time point 10 min after the addition of 3000 units of *Eco*RI enzyme to the residual reaction mixture left after the removal of the sample for the penultimate time point. For some experiments, the *Sal*I digest on λ401 (2) DNA was carried out after the *Eco*RI reaction; samples (20 μl) were withdrawn at timed intervals from the mixture of *Eco*RI restriction enzyme and the intact DNA from λ401 (2) and added to vials at 80°C that contained 10 μl of 0.1 M-Tris/HCl/0.2 M-NaCl/0.01 M-$MgCl_2$, pH 7.5. This vials were cooled and then incubated for 30 min at 37°C after the addition of 10 units of *Sal*I enzyme: the *Sal*I digest was complete before 10 μl of stop solution was added to each vial. With both methods, the samples of stopped reaction mixtures were heated to 67°C and then quenched on ice before loading the entire sample volume onto one slot of a 1% (w/v) agarose-slab gel. The conditions for the electrophoresis of DNA through agarose, the location of the DNA bands on the gel, the measurement of the radioactivity in each band of DNA at each time point during the reaction, and hence the timecourse of product formation, were all as described previously (Halford *et al.*, 1979). Kinetic experiments with the DNA from λ416 (5) or λ421 (4) were carried out similarly to the above, except that the *Sal*I digest was omitted.

For all kinetic experiments, the volume of *Eco*RI enzyme added to the 300 μl reaction mixtures was kept within the range 2–10 μl, pre-dilutions of the enzyme being used so that the reaction rates could be measured on the 0–30 min time scale, i.e. with apparent rate constants between 0.1 and 0.5 min^{-1}. The range of *Eco*RI enzyme concentrations varied from 0.1 nM for the fastest reactions to 10 nM for the slowest. The DNA concentrations were generally held constant at 0.5 nM. Comparisons between the rates of reactions under different conditions or with different substrates were then made after correcting for the concentration of enzyme. For each reaction studied, the rate of that reaction was found to be linearly dependent upon the enzyme concentration: this relationship is reported in more detail by Halford & Johnson (1980). In addition to the specified components, the reaction mixtures also contained the enzyme storage buffer diluted by a factor of 30–150 and the DNA storage buffer diluted by a factor of 2.

Kinetic theory

The *Eco*RI restriction endonuclease catalyses two

consecutive reactions at its recognition site on double-stranded DNA:

$$A \xrightarrow{k_a} B \xrightarrow{k_b} C \qquad (1)$$

where A is the intact DNA duplex, B the first product in which one strand of the DNA has been cleaved at the recognition site and C the final product where both strands of the recognition site have been cleaved (Halford *et al.*, 1979). For a reaction in which the initial substrate (A) is a linear DNA molecule and where product formation is monitored by the electrophoretic mobility of the DNA, then only the final product (C) can be detected unless the DNA is denatured. The nicked product (B) will have the same mobility as the initial substrate (A) if the electrophoresis is carried out on double-stranded DNA. In all of the experiments in this paper, we have monitored the formation of products from linear substrates in their double-stranded form and thus these measurements refer only to the final product, (C). The steady-state kinetics of the conversion of A into C by the *Eco*RI enzyme cannot be analysed by initial rate measurements; the rate of formation of C will be neither linear with time nor directly dependent upon the concentration of A.

We have therefore used integrated rate equations to analyse the entire time-course of the reactions. The necessary controls were described by Halford *et al.* (1979). When $[S] \ll K_m$ so that the time course of product formation in an enzyme-catalysed reaction follows an exponential progress curve, the formation of the final product C from the sequential reactions in eqn. (1) is given by:

$$[C_t] = [C_\infty] \cdot \left\{ 1 + \left[\frac{1}{k_a - k_b} \cdot (k_b e^{-k_a t} - k_a e^{-k_b t}) \right] \right\} \qquad (2)$$

where k_a and k_b are the apparent first-order rate constants for each stage in eqn. (1), $[C_t]$ and $[C_\infty]$ the concentrations of C at time t and time ∞; the boundary conditions for eqn. (2) were noted by Halford (1974). For a system obeying eqn. (2), a semi-logarithmic plot of $\ln([C_\infty] - [C_t])$ against time will be initially curved but subsequently linear at higher time points. A typical semi-logarithmic plot of our experimental data is shown in Fig. 2(*b*). The gradient of the linear portion of the plot is determined exclusively by one from either k_a or k_b, whichever is the smaller. In the cleavage of double-stranded DNA by the *Eco*RI restriction endonuclease, the value of k_a was found to be greater than k_b by a factor of 2 (Halford *et al.*, 1979). This result was obtained with both plasmid pMB9 and λ421 (4) DNA as substrates for *Eco*RI and we have since confirmed these observations with plasmid pAT153 and λ416 (5) DNA. The ratio of two for k_a/k_b has been assigned to the statistical factor arising from the double-stranded nature of DNA. Thus the gradient of the linear portion in a semi-logarithmic plot as Fig. 2(*b*) yields the value for k_b alone. However, one source of error in determining k_b by this method is that if the time required to complete the curvature of the semi-logarithmic plot is underestimated, the value recorded for k_b will be lower than the true value. Mean values of k_b from two or more determinations are given here.

In examining the relationship between k_b and the reaction mechanism of the *Eco*RI restriction enzyme, we consider first reactions carried out at an initial substrate concentration, $[S_0]$, such that $[S_0] > [E_0]$ and also $[S_0] \ll K_m$. The latter condition is met in our experiments with excess substrate over enzyme because the values of k_b were then found to be invariant with increasing DNA concentrations up to 5 nM, the highest concentrations tested here. [Published values for K_m cover the range 3–30 nM for different DNA substrates.] For the minimal reaction scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad (3)$$

the basic steady-state equation is:

$$-\frac{d[S]}{dt} = \frac{k_{cat.}[E_0][S_0]}{[S_0] + K_m}$$

which simplifies under our conditions to:

$$-\frac{d[S]}{dt} = \frac{k_{cat.}[E_0][S]}{K_m}$$

Thus the first-order rate constant for the appearance of product, k_b, is given by:

$$\frac{k_b}{[E_0]} = \frac{k_{cat.}}{K_m}$$

The parameters $k_{cat.}$ and K_m are related to the individual rate constants of the reaction scheme in eqn. (3) and their substitution into eqn. (4) yields:

$$\frac{k_b}{[E_0]} = k_1 \cdot \frac{k_2}{k_{-1} + k_2} \qquad (5)$$

In the above analysis, the observed product corresponds to free product because the concentration of enzyme-bound product must be negligible at low $[E_0]$. But in reactions carried out with a total enzyme concentration, $[E_0]$, such that $[E_0] > [S_0]$ and also $[E_0] \ll K_m^*$, where K_m^* is defined by the enzyme concentration at which the single-turnover rate is one-half of its maximal rate, the observed product could be either enzyme-bound or free and thus we examine the partial mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \qquad (6)$$

For this scheme, the exponential describing product formation (k_b) is given by:

$$k_b = \frac{k_2[E_0]}{[E_0] + K_m^*} \quad (7)$$

where K_m^* is equal to $(k_{-1} + k_2)/k_1$. The formation of free product by dissociation from the EP complex will not affect the observed rate of product formation in eqn. (6). The condition $[E_0] \ll K_m^*$ is met in our experiments because the measured values of k_b were linearly dependent upon $[E_0]$. Hence eqn. (7) simplifies to:

$$\frac{k_b}{[E_0]} = k_1 \cdot \frac{k_2}{k_{-1} + k_2} \quad (8)$$

The identity of eqns. (5) and (8) demonstrate that the kinetic variable measured here, $k_b/[E_0]$, may be equated to $k_{cat.}/K_m$ regardless of whether the data was obtained in a reaction with excess enzyme over substrate or vice-versa.

However, the parameter $k_{cat.}/K_m$ determined in these experiments from measurements of k_b refers only to the second stage of the *Eco*RI-catalysed reaction in eqn. (1), namely the conversion of the nicked DNA to the final DNA product cut in both strands; i.e. S and P in eqns. (3) and (6) refer respectively to B and C in eqn. (1). In addition our values of $k_{cat.}/K_m$ for the conversion of this DNA substrate into product are apparent values that refer to a particular concentration of Mg^{2+}, generally 15 mM in these experiments; the interaction between the binding of Mg^{2+} and of DNA to the *Eco*RI restriction endonuclease is described by Halford & Johnson (1980).

Results

DNA from λ401 (2) and λ416 (5)

To examine the reactions of the *Eco*RI restriction endonuclease at different recognition sites on DNA, we have prepared a series of DNA molecules from derivatives of phage λ that each carry one of the five *Eco*RI recognition sites present on the DNA from λ^+ (Murray & Murray, 1974; see Fig. 1). The DNA molecules from λ416 (5) and λ421 (4) are identical except for the *Eco*RI recognition sites and a single point mutation in gene S of λ. The DNA from λ401 (2) carries a different deletion but is otherwise similar. As Thomas & Davis (1975) reported that the first cleavage of λ^+ DNA occurred at *sr*I 5 at the highest frequency and at *sr*I 2 among the lowest, we commenced our studies by comparing the reactivity of the *Eco*RI restriction enzyme against the DNA from both λ401 (2) and λ416 (5). We found no intrinsic difference in the reactivity of the *Eco*RI enzyme towards the individual recognition sites, *sr*I 2 on DNA from λ401 (2) and *sr*I 5 on DNA from λ416 (5). The differences that were observed between recognition sites were found to be a consequence of the solutions in which the reactions were carried out.

In one set of experiments, rate constants (k_b) from the reaction of the *Eco*RI endonuclease on the DNA of either λ401 (2) or λ416 (5) were measured in solutions that differed only in the concentration of NaCl, the other components of the reaction mixtures (buffer, $MgCl_2$ and pH) being held constant. From these data, shown in Fig. 3, identical reaction rates ($k_b/[E_0]$) were observed with the two substrates in the absence of NaCl. But the manner in which the reactivity of the *Eco*RI restriction enzyme varied with added NaCl differed from one recognition site to the other. Against the DNA from λ401 (2), the enzyme reached maximal activity at 50 mM-NaCl and declined at higher NaCl concentrations, but against the DNA from λ416 (5), maximal activity was not reached until 150 mM-NaCl; at the latter salt concentration, a 10-fold difference in the values of $k_b/[E_0]$ (or $k_{cat.}/K_m$) was noted between the two DNA molecules.

To determine whether these differences were due specifically to NaCl, or were a general ionic-strength effect, the reactions were studied under conditions identical with those in Fig. 3 except that the NaCl

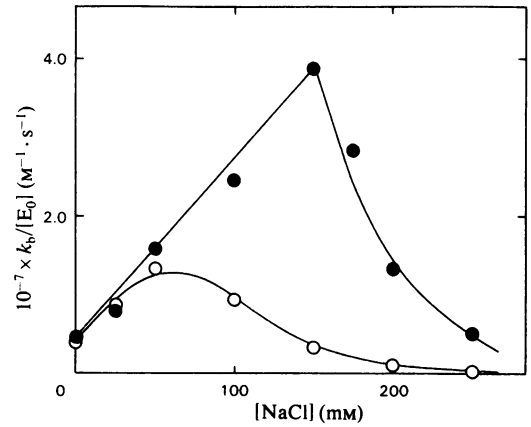

Fig. 3. *Ionic strength dependency*
Rate constants (k_b) were measured from reactions containing the concentration of NaCl indicated and also the *Eco*RI restriction endonuclease with 0.5 nM-DNA (^{3}H-labelled) in 50 mM-Tes/15 mM-$MgCl_2$/0.01% gelatin, pH 7.0, at 22°C: ○, DNA from λ401 (2) after digestion with *Sal*I; ●, DNA from λ416 (5). The reported values of $k_b/[E_0]$ were calculated from the measurements of k_b (between 0.10 and 0.46 min^{-1}) and the total concentration of the *Eco*RI enzyme (between 0.19 and 9.5 nM) for each separate reaction of the type in Fig. 2 and are an average from two or more experiments.

was replaced by either NH_4Cl or sodium acetate. With the DNA from λ416 (5), values for $k_b/[E_0]$ were measured as 2.20, 2.26 and 1.74 nM^{-1}·min^{-1} in 150 mM-NaCl, -NH_4Cl and -sodium acetate respectively. With the DNA from λ401 (2), the values were 0.20, 0.15 and 0.36 nM^{-1}·min^{-1} in the same three 150 mM salt solutions. Thus the difference between the *Eco*RI reactivities on the two substrates at 150 mM-NaCl (Fig. 3) is not due to specific effects by either Na^+ or Cl^- ions, but appears to be a general ionic-strength effect caused by all of the monovalent salts tested.

However, alterations in the concentration of $MgCl_2$ did not affect the different reactivities of the *Eco*RI restriction enzyme against the DNA from λ401 (2) and λ416 (5). The activity of the enzyme against both substrates was found to increase with increasing concentrations of $MgCl_2$ up to a maximum between 10 and 15 mM, but further increases in the $MgCl_2$ concentration caused a decrease in enzyme activity. In one set of experiments carried out at 50 mM-NaCl, the values of k_b on the DNA from λ416 (5) were found to be higher by a constant factor of 1.2 ± 0.2 than those with the DNA from λ401 (2) as the concentration of $MgCl_2$ was varied from 1.0 to 15 mM, with all other conditions as in Fig. 3. Equivalent results were obtained in another set of experiments at 150 mM-NaCl except that here the constant factor between the rates with DNA from λ416 (5) and λ401 (2) was 10 ± 3, but again independent of the $MgCl_2$ concentration.

The reactivity of the *Eco*RI restriction endonuclease towards the DNA from both λ401 (2) and λ416 (5) was also examined as a function of pH (Fig. 4). These experiments were carried out with a constant NaCl concentration of 150 mM, conditions that result at pH 7.0 in a large difference between the reaction rates on the two substrates (Fig. 3). However the difference between these substrates varied across the pH range examined in Fig. 4, as the pH profile for the *Eco*RI reaction with one substrate was distinct from that with the other substrate. With the DNA from λ401 (2), an optimum in $k_{cat.}/K_m$ for the *Eco*RI enzyme was observed at pH 7.5, but with the DNA from λ416 (5) a broad optimum in $k_{cat.}/K_m$ for the same enzyme was found to be centred close to pH 7.0. The experiments in Fig. 4 were not extended to pH values above 8.0 because of the altered specificity of *Eco*RI at pH 8.5 (Polisky *et al.*, 1975).

In all of the above experiments with the DNA from λ401 (2), the substrate for the *Eco*RI reaction was not the intact λ DNA molecule containing only *sr*I 2 but rather fragments of DNA obtained from a *Sal*I digest of DNA from λ401 (2), one of which contains *sr*I 2. However the following experiment demonstrated that the *Eco*RI restriction enzyme reacts identically at *sr*I 2 on these two forms of the substrate. The rate constant of product formation (k_b) was measured in two reactions containing the *Eco*RI restriction enzyme (0.48 nM) with λ401 (2) DNA (0.35 nM) in 50 mM-Tris/HCl/150 mM-NaCl/5 mM-$MgCl_2$/0.01% gelatin, pH 7.7, at 22°C. For one reaction, the DNA had been digested with *Sal*I before the *Eco*RI reaction and a value of 0.12 min^{-1} was measured for k_b. For the other reaction, the intact DNA from λ401 (2) was used as the substrate for the *Eco*RI reaction, which was followed by a *Sal*I digest and here a value of 0.13 min^{-1} was measured for k_b.

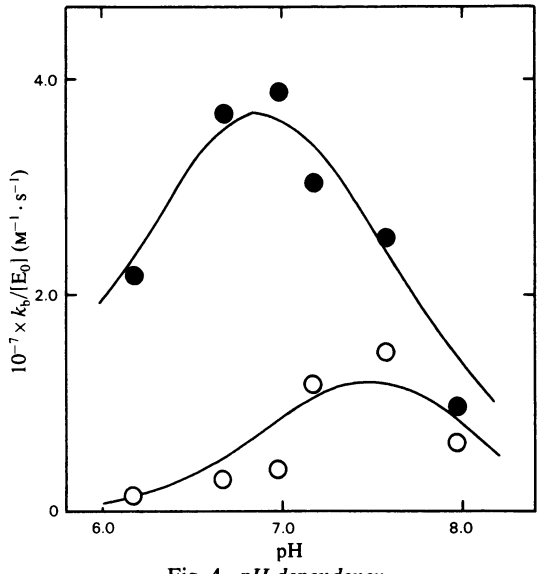

Fig. 4. *pH dependency*

Rate constants (k_b) were measured from reactions at the pH value indicated containing the *Eco*RI restriction endonuclease with 0.5 nM-DNA (^{3}H-labelled) in 50 mM-Tes/150 mM-NaCl/15 mM-$MgCl_2$/0.01% gelatin at 22°C: ○, DNA from λ401 (2) after digestion with *Sal*I; ●, DNA from λ416 (5). The reported values of $k_b/[E_0]$ were calculated from the measurements of k_b (between 0.10 and 0.62 min^{-1}) and the total concentration of the *Eco*RI enzyme (between 0.10 and 4.8 nM) for each separate reaction of the type in Fig. 2 and are an average from two or more experiments. The lines drawn are the theoretical lines from the model:

$$EH_2^{2+} \rightleftharpoons EH^+ + H^+ \rightleftharpoons E + 2H^+$$

where only the EH^+ form of the enzyme has catalytic activity and with the following parameters: for the lower line, $k_{cat.}/K_m$ for $EH^+ = 3.33 \times 10^7$ M^{-1}·s^{-1}, $pK_1 = 7.4$, $pK_2 = 7.5$; for the upper line, $k_{cat.}/K_m$ for $EH^+ = 5.0 \times 10^7$ M^{-1}·s^{-1}, $pK_1 = 6.2$, $pK_2 = 7.5$.

DNA from λ401 (4) and λSH1

Thomas & Davis (1975) reported that the

frequency with which the first *Eco*RI cleavage of λ^+ DNA occurred at *sr*I 4 was in between those at *sr*I 5 and *sr*I 2. Thus we also examined the kinetics of the *Eco*RI restriction endonuclease at *sr*I 4 on the DNA from λ421 (4). However under all conditions tested, which covered the range of solutions used in Figs. 3 and 4, the values of k_b measured from the *Eco*RI reaction on the DNA from λ421 (4) were found to be identical, within the limits of experimental error, with those obtained with the DNA from λ416 (5). The close similarity of the reaction rates of *Eco*RI cleavage on the DNA from λ421 (4) and λ416 (5) was observed both in separate reaction mixtures, each containing one of these substrates, and also in reaction mixtures containing both substrates together.

We considered that a possible cause of the discrepancy between our observations and those of Thomas & Davis (1975) might be the fact that the earlier work had been done on the DNA from λ^+, which contains the five *Eco*RI recognition sites, whereas our studies had employed mutant derivatives of λ whose DNA each carried a single *Eco*RI recognition site. Thus we constructed a derivative of bacteriophage λ, λSH1 (4,5) which contained both *sr*I 4 and *sr*I 5 but no other *Eco*RI recognition sites. Some of the products from the reaction of the *Eco*RI restriction enzyme on the DNA from λSH1 can readily be separated from the substrate by agarose-gel electrophoresis. These are the two final products of mol.wts. 3.73×10^6 (the DNA between *sr*I 4 and *sr*I 5 on λ^+) and 2.13×10^6 (the DNA between *sr*I 5 and the right-hand terminus of λ) and also a partial digest product of mol.wt. 5.86×10^6

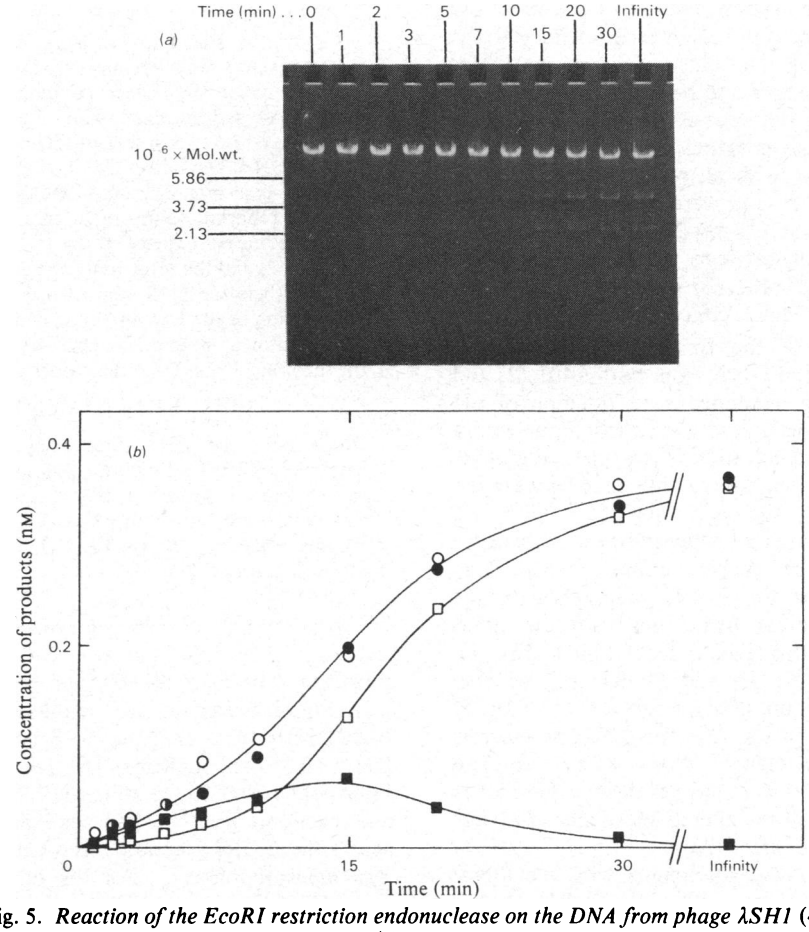

Fig. 5. *Reaction of the EcoRI restriction endonuclease on the DNA from phage λSH1 (4, 5)*
(*a*) Analysis by electrophoresis through a 1.0% (w/v) agarose gel of samples, withdrawn at the times (in min) specified by each slot on the gel, from a reaction mixture containing 0.29 nM-*Eco*RI restriction endonuclease and 0.36 nM-DNA (^{3}H-labelled) from phage λSH1 (4,5) in 50 mM-Tris/HCl/90 mM-NaCl/2 mM-$MgCl_2$/0.01% gelatin, pH 7.5, at 22°C. (*b*) Concentrations of the final products of mol.wt. 2.13×10^6 (○) and of mol.wt. 3.73×10^6 (□), the partial digest product of mol.wt. 5.86×10^6 (■) and the sum of the concentration of the latter two species (●), during the reaction in (*a*).

(obtained after cleavage at *sr*I 4 but not at *sr*I 5). The partial digest product will eventually yield the two final products upon cleavage at *sr*I 5. Thus every DNA cleavage that occurs at *sr*I 5 liberates the final product of mol.wt. 2.13×10^6, and the rate of the reaction at this site on the DNA from λSH1 (4,5) can be measured from the concentration of this product alone. However the cleavage at *sr*I 4 will liberate either the 5.86×10^6 mol.wt. partial digest product or the 3.73×10^6 mol.wt. final product, depending upon whether that particular DNA molecule has been cleaved previously at *sr*I 5; hence, the rate of the reaction at *sr*I 4 is given by the concentrations of both the 5.86×10^6 mol.wt. partial product and the 3.73×10^6 mol.wt. final product summed together.

An analysis of the products formed during the reaction of the *Eco*RI restriction enzyme on the DNA from λSH1 (4,5) is shown in Fig. 5(*a*) and the changes with time in the concentrations of each of the separated products from this reaction in Fig. 5(*b*). As the time course for the appearance of the final product of mol.wt. 2.13×10^6 is indistinguishable from the sum of the partial product of mol.wt. 5.86×10^6 plus the final product of mol.wt. 3.73×10^6, the rates for the *Eco*RI reaction on the DNA from λSH1 (4,5) are the same at both *sr*I 4 and *sr*I 5. The experiment with DNA from λSH1 (4,5) in Fig. 5 was repeated under a variety of experimental conditions covering a range of NaCl concentrations and pH values, but in no instance was any difference beyond experimental error observed between the reaction rates at *sr*I 4 and *sr*I 5. Thus the *Eco*RI restriction endonuclease reacts identically with the recognition sites at *sr*I 4 and *sr*I 5 on λ DNA regardless of whether both sides are on the same DNA molecule or each site is on a separate DNA molecule.

Discussion

The *Eco*RI restriction endonuclease cleaves double-stranded DNA specifically at its recognition site but, even though the same nucleotide sequence is present within each recognition site, different recognition sites on the same DNA molecule may be cleaved by *Eco*RI at markedly different rates. In the present study, we have extended the observations of Thomas & Davis (1975) on the *Eco*RI recognition sites of λ DNA. We found that there was no fundamental difference between the reactivities of the *Eco*RI restriction enzyme towards individual recognition sites on this DNA, for under certain experimental conditions the same reaction rate was noted at each site. But the manner in which the reactivity of the enzyme was altered by changing the experimental conditions differed from one recognition site to another, thus creating large differences between the reaction rates at certain sites (Figs. 3 and 4).

Many enzymes that interact with a number of different sites on one DNA molecule exhibit 'processive' mechanisms where the enzyme catalyses a number of consecutive reactions while remaining associated with the same molecule of DNA (Jovin, 1976). Therefore it is tempting to suggest that the different susceptibilities of the *Eco*RI recognition sites on λ DNA are due to a processive mechanism where the enzyme binds to the DNA at, or close to, the most susceptible recognition sites (*sr*I 4 and *sr*I 5) near the right-hand terminus of λ DNA and that the enzyme then travels along the DNA cleaving each recognition site as it is located. However, the same reactivity is displayed by this enzyme at *sr*I 2 on both the intact DNA from λ401 (2) and the DNA fragment obtained by digesting λ401 (2) DNA with the *Sal*I restriction enzyme; in the latter, the *Eco*RI recognition site is located on a separate DNA molecule from the right-hand segment of λ DNA. In addition, the recognition sites *sr*I 4 and *sr*I 5 are cleaved at identical rates both when the two sites are on the same DNA molecule and also when each site is on a separate DNA molecule (Fig. 5). Moreover, given that the *Eco*RI enzyme can dissociate freely from the DNA duplex after cleaving the first of the two strands within a single recognition site (Halford *et al.*, 1979), it is improbable that the enzyme would remain associated with the DNA for the time interval between the cleavage of the second strand at one recognition site and the first strand at the next recognition site. Thus all of the available data indicate that the cleavage of λ DNA by *Eco*RI is not processive and that the interaction between this restriction enzyme and each recognition site on a DNA molecule is an independent process.

We have also examined the possibility that the different susceptibilities of *Eco*RI recognition sites on λ DNA may result from the accessibility of each site within the three-dimensional structure of the DNA. But it is now generally considered that linear DNA molecules adopt a worm-like coil configuration in solution; as the chain length of λ DNA at over 40000 base pairs is much longer than the persistence length of DNA at about 200 base pairs, the degree of flexibility in λ DNA will be large enough to generate an almost completely random structure (Bloomfield *et al.*, 1974). Thus the probability that any given segment of the DNA chain will be located on the surface of the excluded volume of that DNA molecule is the same for all segments of the DNA.

Therefore we consider the most plausible explanation for the different *Eco*RI reactivities at the individual recognition sites on λ DNA to be that the enzyme interacts with nucleotides both within and adjacent to the recognition site. At present, the

nucleotide sequences adjacent to the *Eco*RI recognition sites on λ DNA are not known. The elucidation of the sequences adjacent to *sr*I 2, *sr*I 4 and *sr*I 5 might enable one to specify which nucleotides outside of the recognition site are involved in these interactions. At two of these recognition sites (*sr*I 4 and *sr*I 5), the enzyme displays the same reactivity, but at the other site (*sr*I 2) a lower reactivity is found under certain conditions and it may be possible to correlate this with homologies and differences between the adjacent sequences. But even when the sequence data and also the crystal structure of the *Eco*RI restriction enzyme become available (Rosenberg *et al.*, 1978), the molecular basis for the different reactivities at these recognition sites may still not be apparent. The 10-fold difference in the $k_{cat.}/K_m$ values at *sr*I 2 and *sr*I 5 (Fig. 3) corresponds to a difference in activation energy of only 5.6 kJ/mol. This change of activation energy is small enough to arise from the formation of a single hydrogen bond in the transition state of the reaction at *sr*I 5 which is not formed during the reaction at *sr*I 2.

However it is possible to account for the reactivities of the *Eco*RI restriction enzyme at these recognition sites in terms of a physical mechanism that can accommodate the effects of both salt and pH on these reactions. We consider first the pH-dependency of $k_{cat.}/K_m$, at constant NaCl concentration, from the *Eco*RI reactions on both the DNA from λ401 (2) and λ416 (5); see Fig. 4. The bell-shaped pH profiles with both substrates can be fitted theoretically to two ionizing groups on the enzyme:

$$EH_2^{2+} \overset{K_1}{\rightleftharpoons} EH^+ + H^+ \overset{K_2}{\rightleftharpoons} E + 2H^+ \qquad (9)$$

where the species EH^+ is the only active form of the enzyme but that this reacts slowly with the substrate compared with the rates of the protonation equilibria. (The substrate in these kinetic experiments is the DNA in which one strand has been cleaved and thus the second pK_a of the phosphomonoester may also affect the reaction.) This model yields similar values of $k_{cat.}/K_m$ for the species EH^+ on both substrates, identical values for the pK_a of the group responsible for the alkaline side of the bell-shaped plots (pK_2) but very different values for the pK_a of the group on the acidic side (pK_1); the values are given in Fig. 4. However, the interpretation of pH dependencies of steady-state kinetics in terms of ionizing groups is only one of many possible interpretations (Knowles, 1976). It is possible that the pK_a of 7.5 observed with both substrates on the alkaline side of the pH profile reflects a real ionization of a group on the enzyme, but difficulties are encountered in relating the observed pK_a values on the acidic side to ionizing groups. Firstly, these pK_a values are not the same for both substrates, best fits being obtained with $pK_1 = 6.2$ for the reaction on λ416 (5) DNA and with $pK_1 = 7.4$ on λ401 (2) DNA. As the pH dependency of $k_{cat.}/K_m$ reflects ionizations within the free enzyme and not the enzyme–substrate complex (Peller & Alberty, 1959), this discrepancy cannot arise from the same group undergoing different pK_a perturbations in the two enzyme–substrate complexes. The different values for pK_1 would have to refer to different ionizing groups, which leads to the unacceptable conclusion that a group with pK_a 7.4 is involved in the reaction on λ401 (2) DNA but not in that on λ416 (5) DNA. Secondly, at pH 7.0 in 150 mM-NaCl, identical values for the equilibrium dissociation constant of the *Eco*RI restriction enzyme, measured in the absence of Mg^{2+}, were obtained from both DNA molecules (Halford & Johnson, 1980).

Thus it seems likely that the pK_a values on the acidic side of the pH profile in Fig. 4 are apparent pK values rather than true ionization constants. From the description of $k_{cat.}/K_m$ in terms of the individual rate constants of the enzyme mechanism in eqn. (3), it follows from eqns. (5) and (8) that:

$$\frac{k_{cat.}}{K_m} = k_1 \qquad \text{when} \qquad k_2 \gg k_{-1}$$

and

$$\frac{k_{cat.}}{K_m} = k_1 \cdot \frac{k_2}{k_{-1}} \qquad \text{when} \qquad k_2 \ll k_{-1}$$

Hence, if the value of k_2 changes from below to above that of k_{-1} as the pH is raised, the pH dependency of $k_{cat.}/K_m$ will follow an ionization curve with an apparent pK_a value at the pH where $k_2 = k_{-1}$ (Renard & Fersht, 1973). We assume here that the rate constant k_1 will be the same for the reactions on the DNA from both λ401 (2) and λ416 (5); the bimolecular rate is probably diffusion-controlled (Richter & Eigen, 1974) and the two DNA substrates have very similar physical properties. It then follows from the identical values of the equilibrium dissociation constant from both DNA molecules (Halford & Johnson, 1980) that the rate constant k_{-1} will also be the same in the reactions on each substrate. Thus, on the acidic side of the pH profile of $k_{cat.}/K_m$ for the *Eco*RI enzyme, the different values for pK_1 can be accounted for by the value of k_2 increasing with increasing pH, but that at all pH values in this range k_2 is greater by a factor of (log 1.2) in the reaction on the DNA from λ416 (5) than it is for that on λ401 (2) DNA. The deviation of 1.2 pH units between the apparent pK_a values for the two reactions in 150 mM-NaCl is then due to k_2 being faster than k_{-1} in the reaction on λ416 (5) DNA at pH values above 6.2, but that k_2 becomes faster than k_{-1} in the reaction on λ401 (2) DNA only at pH values above 7.4.

This mechanism for the pH dependencies can also accommodate the different variations with NaCl concentration in $k_{cat.}/K_m$ for the *Eco*RI restriction enzyme on the DNA from both λ401 (2) and λ416 (5) at pH 7.0; see Fig. 3. It is to be expected that the value of k_{-1} will be decreased by decreasing the NaCl concentration of the reaction mixture from 150 to 50mM, as this decrease in ionic strength would stabilize electrostatic interactions between the protein and DNA. The equilibrium dissociation constants between the *Eco*RI restriction enzyme and both λ DNA derivatives are lower at 50mM than at 150mM (Halford & Johnson, 1980). With a different system, the rate constant for the dissociation of the *lac* repressor from its operator is decreased at low ionic strength (Riggs *et al.*, 1970). Thus at ionic strengths below 50mM-NaCl and at pH 7.0, we predict that k_{-1} is smaller than k_2 for the *Eco*RI reactions on the DNA from both λ401 (2) and λ416 (5) so that, even though the values of k_2 differ from one substrate to another, the parameter $k_{cat.}/K_m$ will be equal to k_1 with both substrates. Consequently the two substrates yield similar values for $k_{cat.}/K_m$ at low ionic strength. But, as the ionic strength is increased, the inequality $k_{-1} < k_2$ will hold for the reaction of λ416 (5) DNA up to a higher ionic strength than will be case for λ401 (2) DNA, because k_2 is greater in the *Eco*RI reaction on λ416 (5) DNA than it is on λ401 (2) DNA. Thus, the concentration of NaCl beyond which further increases in NaCl cause a decrease in $k_{cat.}/K_m$ will be at a higher salt concentration for the reaction on λ416 (5) DNA than on λ401 (2) DNA. This accounts for the observed maximal activities of the *Eco*RI enzyme at 150mM-NaCl with the DNA from λ416 (5) and at 50mM-NaCl with λ401 (2) DNA.

By assigning the kinetic basis for the different reactivities of the *Eco*RI restriction endonuclease at these recognition sites to the rate constant (k_2) for the conversion of the enzyme–substrate complex to the enzyme–product complex, it follows that the interactions between the enzyme and DNA sequences adjacent to the recognition site (which we suggest form the molecular basis for these differences) occur only at the transition state of the cleavage reaction and not on the initial binding of the enzyme to the DNA.

Regardless of the mechanism by which the differences in the reactivity of the *Eco*RI restriction endonuclease towards individual recognition sites are generated from changes in the reaction conditions, these differences are of sufficient magnitude to permit their exploitation. Class II restriction enzymes have found wide application in the study of DNA and in the construction of recombinant DNA molecules (Roberts, 1976). For certain experiments in this field, it is required that the restriction enzyme cleave every recognition site on the DNA. Experiments of this type should therefore be carried out under conditions where the restriction enzyme displays its maximal activity against its least susceptible site. For the *Eco*RI enzyme, this requirement is met by the conditions of 50mM-NaCl, pH 7.5, which are the standard conditions employed with this enzyme (Greene *et al.*, 1974). But in other experiments, the desired products are DNA fragments that have been cleaved at a limited number of recognition sites (for example, Maniatas *et al.*, 1978). However the premature termination of a restriction enzyme digest under the standard conditions will produce a random collection of partial digest products. Yet by carrying out the reaction under conditions where certain sites are cleaved much faster than others (150mM-NaCl, pH 6.5, for the *Eco*RI enzyme) and then terminating the reaction at the appropriate time, it will be possible to obtain a specified set of partial digest products.

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