Biochem. J. (1983) **211**, 405–415
Printed in Great Britain

Single turnovers of the *Eco*RI restriction endonuclease

Stephen E. HALFORD and Nicola P. JOHNSON
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(*Received 1 November 1982/Accepted 11 January 1983*)

Single turnovers of the *Eco*RI restriction endonuclease, cleaving its recognition site on the covalently closed form of plasmid pMB9, were examined. Two methods were used to monitor the progress of the reactions: one involved quenching the reaction at various times followed by the electrophoretic separation of the products cleaved in one and in both strands of the duplex; the other employed a stopped-flow fluorimeter to measure the amount of ethidium bromide bound to the DNA as it changes when the DNA, cleaved in at least one strand, dissociates from the enzyme. Two procedures were used to initiate the reactions. For some, one solution containing the enzyme was mixed with a second containing both DNA and $MgCl_2$: in these reactions, the fluorescence changed at the same rate as the cleavage of the first strand of the duplex. Other reactions were started by the addition of $MgCl_2$ to a pre-equilibrium of enzyme and DNA: here, both strands of the DNA were cleaved faster than before, with the fluorescence signal now occurring at the same time as the cleavage of the second strand. The different kinetics from the two assays and the two mixing procedures are consistent with the rates of these reactions being controlled by protein conformational changes. These may affect either one subunit alone within the dimeric *Eco*RI enzyme, allowing the enzyme to cleave only one strand of the DNA in each turnover. Alternatively, both subunits of the dimer may change, so that the enzyme then cleaves both strands during the life-time of one enzyme–DNA complex.

The *Eco*RI restriction endonuclease (EC 3.1.23.13) recognizes the symmetrical sequence

```
      ↓
5'-G-A-A-T-T-C-3'
3'-C-T-T-A-A-G-5'
            ↑
```

on duplex DNA and, in the presence of Mg^{2+}, cleaves both strands of the DNA at the sites indicated by arrows (Hedgpeth *et al.*, 1972). Catalytic concentrations of this enzyme exist in solution as dimers of identical polypeptide subunits (Roulland-Dussoix *et al.*, 1974; Modrich & Zabel, 1976). The nucleotide bases and the phosphate groups within and adjacent to the recognition site, whose chemical modification affects the binding of the *Eco*RI restriction enzyme, are disposed symmetrically on both strands of the DNA (Lu *et al.*, 1981). Hence the interaction between the enzyme and its recognition site may involve matching the symmetry between the two protein subunits to that within the DNA sequence at the recognition site, as was first proposed for DNA–protein complexes by Kelly & Smith (1970). On this model, the catalytic site of one protein subunit would presumably be juxtaposed against the scissile phosphodiester bond on one strand of the DNA, and the catalytic site from the second polypeptide subunit similarly placed on the other strand. Certain restriction endonucleases, such as *Bgl*II and *Sal*GI, can cleave both strands of the DNA at their recognition sites within a single enzyme–DNA complex (Imber & Bickle, 1981; Maxwell & Halford, 1982).

In contrast, during the reaction of the *Eco*RI restriction endonuclease, DNA intermediates cut in one strand are observed. From measurements of $k_{cat.}/K_m$ for the cleavage of each strand of the *Eco*RI recognition site, Halford *et al.* (1979) suggested that the enzyme dissociates freely from the DNA after cleaving the first strand and that a separate reaction is required to cleave the second strand. However, in our previous studies on the kinetics of the *Eco*RI enzyme (Halford *et al.*, 1979, 1980; Halford & Johnson, 1981), the reaction mixtures had generally contained low concentrations of the enzyme with the result that, at any given time in the reaction, only a

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

small fraction of the total DNA would be bound to enzyme. In the present investigation we have extended the kinetics of the *Eco*RI restriction enzyme to single turnovers at high concentrations of the enzyme, so that transient intermediates of enzyme–DNA complexes may be detected.

Apart from the hydrolytic reaction, any proposal for the mechanism of the *Eco*RI restriction endonuclease must also describe how processes that occur after the binding of the enzyme to the DNA permit the discrimination between the recognition site and alternative sequences on the DNA. Firstly, the different kinetics for the cleavage of separate *Eco*RI recognition sites on one DNA molecule appear to be caused by interactions with neighbouring nucleotides that affect the rate of hydrolysis of enzyme-bound DNA rather than the affinity of the enzyme for each site (Halford *et al.*, 1980). Secondly, the difference between the K_D value (equilibrium dissociation constant) for the specific binding of the *Eco*RI restriction enzyme to its recognition site, compared with that for non-specific binding at alternative sequences, is not large enough to account for the specificity of this enzyme for DNA cleavages at the recognition site (Halford & Johnson, 1980). [We previously analysed our data on the filter-binding of the *Eco*RI enzyme to DNA by equations that were inappropriate to this technique. Our data have since been re-analysed by Clore *et al.* (1982), whose simplest interpretation was that the *Eco*RI enzyme binds specifically to its recognition site with a K_D of 1 nM and that the non-specific binding to alternative sequences is non-co-operative and has a K_D of 0.15 mM. The latter value, but not the former, differs significantly from that given by Halford & Johnson (1980), and in the present paper we use the values from Clore *et al.* (1982). The revised value of the K_D for non-specific binding agrees with that obtained by Langowski *et al.* (1980) from competitive inhibition studies.]

Methods and materials

The purification of the *Eco*RI restriction endonuclease to greater than 90% homogeneity and its storage were described by Halford *et al.* (1979). Immediately before the reaction, the enzyme was diluted by at least 50-fold into the requisite buffer (usually 50 mM-Tes/NaOH buffer, pH 7.0, containing 50 mM-NaCl, 10 μM-ethidium bromide and 0.01% gelatin, at 22°C). Control experiments showed that the *Eco*RI enzyme retained full activity after 1 h in this diluent. Concentrations of the *Eco*RI restriction enzyme were determined by amino acid analysis and are given in terms of the dimeric protein of M_r 62000 (Newman *et al.*, 1981; Greene *et al.*, 1981). The formation of the tetramer requires protein concentrations above 100 μM (Modrich & Zabel, 1976), higher than any used in the present work.

Deoxyribonuclease from bovine pancreas (lot no. M441; Miles Laboratories, Slough, Berks., U.K.) was dissolved in 10 mM-HCl and stored at −20°C: these solutions were diluted into the buffer required for the reaction, also containing 100 μM-phenylmethanesulphonyl fluoride, immediately before use. The gravimetric concentrations are given.

Plasmid pMB9, either unlabelled or labelled *in vivo* by [*Me*-^{3}H]thymidine, was purified by CsCl centrifugations as described by Halford & Johnson (1981). All experiments described below used DNA preparations in which it was shown by electrophoresis that over 90% of the DNA molecules were covalently closed circles of the monomeric form of plasmid pMB9. Concentrations of plasmid pMB9 were determined by using $A_{260}^{0.1\%} = 20$ and are given in terms of duplex DNA molecules of M_r 3.6×10^6.

The stopped-flow fluorimeter of Dr. D. W. Yates was used to measure ethidium bromide fluorescence as described by Halford & Johnson (1981), apart from some modifications to the system for data capture and analysis. The amplified output from the photomultiplier was now processed by a fast A/D converter before the input of an ITT 2020 computer, which stored 1024 voltage readings across the pre-set time base. In the experimental records shown (Figs. 1*b* and 2), pre-trigger signals of 100 data points were recorded before the start of the reactions and fluorescence enhancements are shown by downward deflexions. Permanent records were made by transferring the data from the computer to discs. The data were analysed by first averaging several records (more than five) of the same reaction and then lowering the number of points on the summed trace to 256 by block-averaging: subsequently, an exponential was fitted by non-linear regression (McCalla, 1967) to all of the data within a selected time range. Simulations were done on a Hewlett–Packard HP 85A computer with programs from Professor H. Gutfreund.

Gel assays on the cleavage of ^{3}H-labelled plasmid pMB9 by the *Eco*RI restriction enzyme were performed by placing a sample (15–25 μl) from a solution of one reagent in a small vial, and holding the vial on a vortex mixer while adding first a sample of equal volume from the solution of the second reagent followed by 25 μl of Stop-Mix (see below). The addition of the second reagent and the Stop-Mix were made by two Gilson air-displacement pipettes, each held by a different person, and the time interval between the two additions was recorded by digital stop-watch. The shortest time interval achieved was 0.5 s. Subsequent handling for the separation of the DNA substrate from the products by gel electrophoresis and the determination of each DNA concentration by scintillation counting of radio-

activity was as described by Halford *et al.* (1979). The Stop-Mix generally used in these experiments was 0.1 M-EDTA/0.1 M-Tris/HCl buffer, pH 8.0, containing 0.02% Bromophenol Blue and 40% (w/v) sucrose. Control experiments were done with other Stop-Mixes: 0.1 M-EDTA/0.5 M-sodium acetate buffer, pH 4.0; 0.1 M-EDTA/0.5 M-glycine/NaOH buffer, pH 11.0; 0.1 M-EDTA/4.0 M-NaCl, pH 8.0. All four Stop-Mixes yielded identical kinetics from the same reaction. Therefore the addition of EDTA to a final concentration above that of $MgCl_2$ terminates catalysis by the *Eco*RI enzyme instantaneously on the time-scales being considered.

The major sources of errors in this gel assay will be due to incomplete mixing of either the two reagents or the Stop-Mix, or to mistakes in timing short intervals (less than 5 s) between adding the second reagent and the Stop-Mix. The effect of these non-systematic errors was minimized by analysing a number of samples quenched at similar times after mixing. For each reaction studied, one pair of reagent solutions was prepared from which the samples of each were taken to yield 32 mixtures: duplicates were run on both the zero time point, t_0 (where the Stop-Mix was added before the second reagent) and the infinity time point ($t_\infty > 300$ s); a further 28 samples of the mixed reagents were quenched at different times in the range 0.5–150 s, with at least eight samples being stopped within the first 5 s. Since it was impossible to quench at identical times when repeating the same reaction, with another pair of reagent solutions of identical composition, the data from different copies of the same reaction were not averaged: instead each was analysed individually. The rate constants were generally obtained from three or more analyses on one reaction and varied by less than 25%.

Results and discussion

Methods of analysis

We have studied the kinetics of single turnovers of the *Eco*RI restriction endonuclease in reactions with excess enzyme over substrate. The substrate, plasmid pMB9, was isolated as a covalently closed circle of duplex DNA and carries one copy of the *Eco*RI recognition sequence (Rodriguez *et al.*, 1976). At the start of our reactions, in buffers containing ethidium bromide, the covalently closed DNA will be positively supercoiled (Halford & Johnson, 1981). The cleavage of one strand of the duplex will yield the relaxed conformation of open-circle DNA, whereas the cleavage of both strands at the same site generates the linear form. Two methods were used to monitor the progress of these reactions, namely gel and fluorescence assays.

In the gel assay, the reaction between the *Eco*RI enzyme and plasmid pMB9 in the presence of $MgCl_2$ was quenched by the addition of EDTA at various times after mixing the reagents. The covalently closed, open-circle and linear forms of the DNA were then separated from each other by gel electrophoresis, and the concentrations of each were determined (Fig. 1*a*). This assay was used previously in a steady-state analysis of the cleavage of plasmid

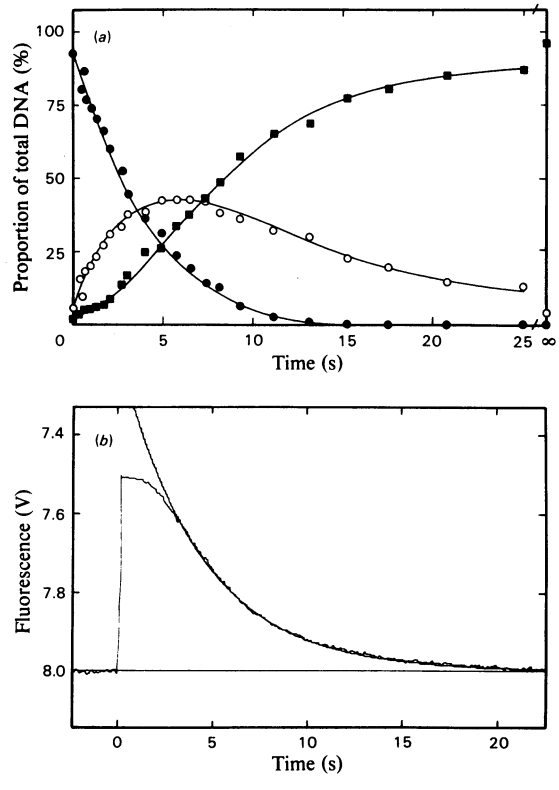


Fig. 1. *Gel and fluorescence assays of EcoRI restriction endonuclease activity*

Reaction mixtures contained *Eco*RI restriction endonuclease (17 nM), plasmid pMB9 (2.0 nM in *a*, 0.28 nM in *b*) and $MgCl_2$ (15 mM) in 50 mM-Tes buffer, pH 7.0, containing 50 mM-NaCl, 10 μM-ethidium bromide and 0.01% gelatin, at 22°C. Before the mixing, enzyme was in one solution, DNA and $MgCl_2$ were in the second and all other components were in both. In (*a*), samples of the reaction were quenched at the times indicated and the concentrations of covalently closed (●), open-circle (○) and linear (■) forms of the DNA are given as percentages of the total DNA. In (*b*), the change in fluorescence (relative to 8 V at the reaction end-point), after mixing the reagents at the indicated time zero, was monitored on the stopped-flow fluorimeter: the Figure shows the average of eight records of the reaction, the base-line fitted to the end-point and the curve of the exponential fitted to all data between 4.4 and 13.4 s.

pMB9 by *Eco*RI restriction endonuclease (Halford *et al.*, 1979), but, because the *Eco*RI restriction enzyme has a low catalytic-centre activity, it was applicable to single turnovers without recourse to rapid reaction techniques. As a first approximation, shown below to be an over-simplification, data such as Fig. 1(*a*) could be described by eqn. (1):

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

In this context, A is covalently closed DNA, B the open-circle form and C the linear form, and k_a and k_b are first-order apparent rate constants. From the equations for the time-dependence of [A], [B] and [C] in eqn. (1) (Capellos & Bielski, 1972), a plot of $\log[A_t]$ against time yields k_a directly. However, a plot of $\log([C_\infty]-[C_t])$ will be curved during the initial lag phase but become linear at later time points (see Fig. 3 below): the gradient of the linear portion yields the smaller of k_a and k_b, but in all of our reactions this gradient was less than k_a and hence gives k_b. The gel assay measures only the total concentrations of the three forms of the DNA and provides no information on whether these are enzyme-bound or free.

The fluorescence assay (Halford & Johnson, 1981) depends on covalently closed DNA binding ethidium with a different stoicheiometry from either the open-circle form or the linear form, both of which bind the same amount of ethidium. At the concentration of ethidium bromide used in the present work (10 μM), less dye would be bound to the covalently closed form of plasmid pMB9 than to either the open-circle form or the linear form. Ethidium has a much higher quantum yield when bound to DNA than when it is free in aqueous solution. Thus the cleavage of at least one strand of the DNA from plasmid pMB9 by the *Eco*RI restriction endonuclease is accompanied by an enhanced fluorescence from ethidium (Fig. 1*b*). The application of this assay to single turnovers of the *Eco*RI enzyme required a stopped-flow fluorimeter, because the time taken to mix the reagents in the cuvette of a conventional fluorimeter would have precluded measurements in the first 10s of the reaction. The covalently closed form of plasmid pMB9 is not sheared by passage through the stopped-flow fluorimeter (Halford & Johnson, 1981).

Both the open-circle form and the linear form of the DNA bind more ethidium than does the covalently closed form because they are free to rotate one strand of the DNA around the other and hence can accommodate the unwinding caused by ethidium intercalation: the topology of covalently closed DNA prevents its strand rotation (Bauer, 1978). After the enzyme has hydrolysed at least one phosphodiester bond on the DNA but while it remains bound to the DNA at the cleavage site, the rotation of the free end of one strand around the other strand of the duplex would be sterically hindered: the free end would still be within the active site of the enzyme. It seems likely that the rotation of the strands could only occur after the enzyme has dissociated from the DNA, and hence the fluorescence enhancement would occur at this stage of the reaction rather than during the hydrolytic step. Experimental results supporting this suggestion are presented below, where it is shown that, during single turnovers of the *Eco*RI restriction enzyme, the fluorescence rises at a later time than the cleavage of the first strand of the DNA. [The data of Halford & Johnson (1981), showing that the fluorescence signal was concomitant with the cleavage of the first strand, were obtained in reactions where only a small fraction of the DNA was enzyme-bound at any one time, so the concentration of a transient enzyme–product complex would have been negligible.]

Under our experimental conditions, ethidium binds rapidly to either covalently closed or relaxed DNA (Halford & Johnson, 1981), but there remains the possibility that the rate of the fluorescence change from reactions such as Fig. 1(*b*) is limited by

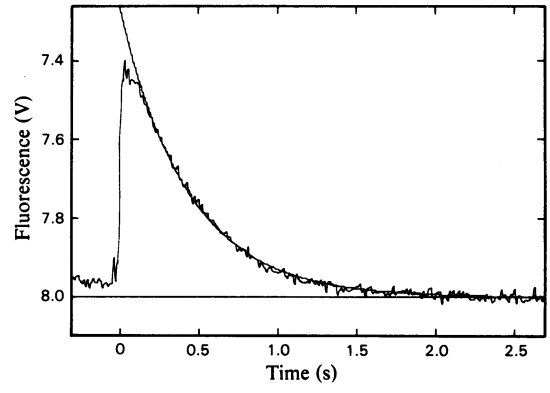


Fig. 2. *Fluorescence and pancreatic deoxyribonuclease*
The reaction mixture contained pancreatic deoxyribonuclease (7 μg/ml), plasmid pMB9 (0.20nM) and $MgCl_2$ (5mM) in 50mM-Tes buffer, pH 7.0, containing 50mM-NaCl, 10 μM-ethidium bromide and 0.01% gelatin, at 22°C. The change in fluorescence (relative to 8V at the reaction end-point) was recorded after mixing in the stopped-flow fluorimeter at the indicated time zero one solution of pancreatic deoxyribonuclease and $MgCl_2$ with a second of DNA, all other components being in both. The Figure shows the average of six records of the reaction, the base-line fitted to the end-point and the curve of the exponential fitted to all data between 0.15 and 1.47s ($k_f = 2.34\,s^{-1}$).

the rate at which the strands of the duplex rotate around each other. To examine this possibility, the fluorescence assay was also used to measure the rate at which pancreatic deoxyribonuclease cleaves plasmid pMB9 (Fig. 2). Fluorescence will detect the first cleavage of a phosphodiester bond by this endonuclease, which could be at any location on the DNA: gel assays showed that the product was open-circle DNA, in agreement with Campbell & Jackson (1980). The reaction was studied over a range of enzyme concentrations from 0.3 to 15 μg/ml, and a single exponential was fitted to the fluorescence data for each reaction. [At high concentrations of pancreatic deoxyribonuclease, such as in Fig. 2, the fluorescence readings during the first 200ms after mixing the reagents deviated from a true exponential.] The first-order rate constants obtained by this procedure varied linearly with the concentrations of pancreatic deoxyribonuclease (results not shown). At the highest concentrations of this enzyme, the rates were much faster than those obtained with the *Eco*RI restriction endonuclease (compare Figs. 1*b* and 2). Thus the rate of the fluorescence change in the reaction catalysed by the *Eco*RI enzyme cannot be limited by the structural alterations to the DNA that are required for binding additional ethidium. The rotation of the strands that accompanies the conversion of supercoiled covalently closed DNA to relaxed open-circle DNA must be a rapid process with a half-time very much less than 1 s.

A typical record of the fluorescence enhancement after the stopped-flow mixing of *Eco*RI enzyme, plasmid pMB9 and $MgCl_2$ in the presence of ethidium bromide is given in Fig. 1(*b*). The record shows a lag phase preceding the enhancement, after which the final 75% of the reaction amplitude closely follows a single exponential: an apparent rate constant, k_f, was evaluated from the exponential phase. We have attempted to simulate data of the type in Fig. 1(*b*) on a sequence of two consecutive reactions, i.e. eqn. (1) where A and B now reflect two compounds with the same low fluorescence and C the reaction product with enhanced fluorescence. No combinations of the values for k_a and k_b gave simulated process curves for the formation of C that resembled the data: with values for k_a and k_b that differed by a factor of more than 3, the simulated lag phases were shorter than those observed; with values of k_a and k_b that differed by a factor of less than 3, the simulated reactions followed exponential curves only during the final 35% (or less) of their amplitudes. However, the progress curve for the formation of product from a sequence of consecutive reactions is determined, not only by the similarity of the rate constants for each step, but also by the number of steps in the sequence (Fuortes & Hodgkin, 1964). Thus we also attempted to simulate our fluorescence records with a sequence of four reactions:

$$V \xrightarrow{k_v} W \xrightarrow{k_w} X \xrightarrow{k_x} Y \xrightarrow{k_y} Z \qquad (2)$$

where Z is the only species with enhanced fluorescence. Among the simulated progress curves for the formation of Z in eqn. (2), some were found to deviate from the experimental curves by less than 2% of the amplitude: the fit was achieved by selecting one rate constant to be equal to the experimental value of k_f and the other three at values between 5 and 10 times greater than k_f (simulations not shown). But attempts to evaluate, as opposed to simulate, several rate constants from data such as Fig. 1(*b*) failed because it was found that many different combinations of values fitted equally well. Moreover, the symmetry in the rate equations for consecutive reactions (Capellos & Bielski, 1972) renders meaningless the assignment of the rate constants used in the simulations to any one of the steps in eqn. (2). Hence we take our stopped-flow fluorescence records to show only that the rate of dissociation of the cleaved DNA from the *Eco*RI enzyme is controlled by a sequence of several consecutive reactions in which the smallest rate constant is equal to k_f.

The quality of the experimental results obtained in gel assays is lower than that from stopped-flow fluorescence, hence precluding the same computer analysis, but it is probable that similar complications would also affect the analysis of gel assays. From the concentration of linear DNA measured by gel assay, the plots of $\log([C_\infty]-[C_t])$ against time were linear over the final 75% of the reaction amplitude (Fig. 3 below), whereas eqn. (1) predicts that, when k_a has a value double that of k_b (as we observe), only the final 25% should produce a linear plot. A similar discrepancy between data and theory was noted in a lack of correlation between the peak amount of open-circle DNA produced during the reaction (Fig. 1*a*) and the relative values of k_a and k_b determined as above, less open-circle DNA being observed than would be expected. Hence it is probable that each step in eqn. (1) should be replaced by a sequence of consecutive reactions (eqn. 2), with k_a and k_b the smallest rate constants in each sequence. [The value of $k_a/[E_0]$ is equal to $k_{cat.}/K_m$ for the enzymic cleavage of the first strand of the DNA, and $k_b/[E_0]$ to $k_{cat.}/K_m$ for the second strand, only in reactions where the concentrations of both enzyme and DNA are below the K_m value (Halford *et al.*, 1979).] Langowski *et al.* (1981) also measured the apparent rate constants for the cleavage of the first and second strands at the *Eco*RI recognition site. However, the apparent rate constants obtained by Langowski *et al.* (1981) are not comparable with ours: firstly, Langowski *et al.*

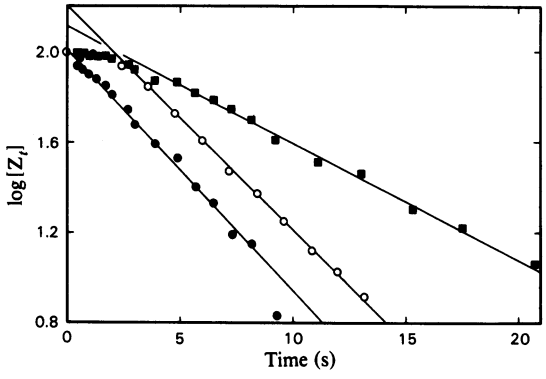


Fig. 3. *Reaction of enzyme against DNA–$MgCl_2$*
Data from the reactions in Fig. 1 are shown in semi-logarithmic form. At time t, $[Z_t]$ is the concentration of either covalently closed DNA (●) or linear DNA (■) in Fig. 1(*a*) or is proportional to the fluorescence enhancement (○) in Fig. 1(*b*): all three have been normalized on a scale from $[Z_0] = 100$ to $[Z_\infty] = 0$. The lines drawn are single exponential fits to all data in the following time spans: covalently closed DNA (●), between 0.9 and 8.2 s, $k_a = 0.25\,s^{-1}$; linear DNA (■), between 4.9 and 20.7 s, $k_b = 0.12\,s^{-1}$; fluorescence (○), between 4.4 and 13.4 s, $k_f = 0.23\,s^{-1}$.

(1981) determined k_b from only the peak of production of the open-circle form via eqn. (1), and thus their values of k_b are too high: secondly, Langowski *et al.* (1981) covered a range of enzyme concentrations from below to above that of the DNA, across which the mechanistic interpretation of k_a and k_b must differ, and this may be the cause of their sigmoidal relationship between the apparent rate constants and the total (rather than the free) enzyme concentration.

Below, we compare the kinetics of product formation from both gel and fluorescence assays in pairs of reactions under identical conditions. For reactions monitored by the gel assay, concentrations of ^{3}H-labelled DNA were typically 2.0 nM, but the more sensitive fluorescence assay used DNA concentrations around 0.2 nM. However, control experiments with both gels and fluorescence showed that, when $[E_0] \gg [S_0]$, the reaction rates were invariant with the DNA concentrations. Fluorescence assays demanded the presence of ethidium bromide in the reaction mixtures, and to facilitate the comparisons, solutions for gel assays also contained ethidium. To initiate the reactions, three procedures were used to mix the *Eco*RI restriction enzyme, plasmid pMB9 and $MgCl_2$. Firstly, enzyme in one solution was mixed with both DNA and $MgCl_2$ in a second solution: this procedure is described as a mode 1 reaction. Secondly, for a mode 2 reaction, a solution containing both enzyme and DNA was added to $MgCl_2$. Thirdly, enzyme and $MgCl_2$ together were mixed with the solution of DNA: here, the kinetics of product formation were identical with those observed in mode 1 reactions of the same final composition, and they are not described further. Thus the differences between mode 1 and mode 2 reactions reported below cannot be due to the location of $MgCl_2$ before mixing. However, in the absence of $MgCl_2$, the *Eco*RI enzyme binds to DNA preferentially at the recognition site (Halford & Johnson, 1980), so that the mode 2 reaction will start from the enzyme–DNA complex whereas mode 1 also involves the binding process.

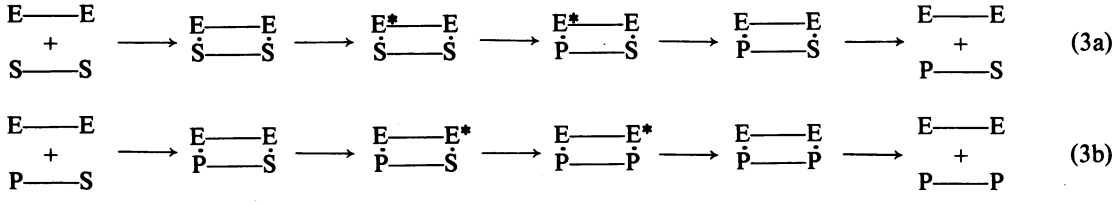
Reactions of enzyme against DNA

Fig. 1 displays a pair of identical mode 1 reactions monitored by either gel or fluorescence assays. Data for these reactions are shown in semi-logarithmic form in Fig. 3. The cleavage of the first strand of the DNA, measured by gel assay from the decreasing concentration of covalently closed DNA, follows a single exponential (k_a) for the complete reaction. But the cutting of the second strand at the recognition site, given on the gel assay by the increasing concentration of linear DNA, involves a lag before an exponential phase: the exponential phase of second strand cleavage yields a rate constant, k_b, whose value is close to one-half of k_a. The fluorescence record also contains a lag phase, of similar duration to that preceding the cleavage of the second strand, followed by an exponential phase for which the rate constant (k_f) has the same value as that for cutting the first strand (k_a).

The observation that the fluorescence record cannot be superimposed upon the time courses for either first or second strand cleavages eliminates the possibility that the binding of additional ethidium to the cleaved DNA is concomitant with the hydrolysis of either phosphodiester. If we are correct in suggesting that the fluorescence is enhanced only when the DNA, cleaved in at least one strand, dissociates from the enzyme, then fluorescence preceding the cleavage of the second strand (Fig. 3, after their lag phases) provides direct evidence that the *Eco*RI enzyme dissociates from the DNA between the two cleavage reactions. In addition, the similarity of the values of k_f and k_a indicate that the slowest rate constant in the sequence of consecutive reactions leading to the dissociation of the nicked DNA (k_f) is the same as that in the sequence of reactions leading to the enzymic hydrolysis of the first strand (k_a). The lag phase in the fluorescence record could be due to processes within the mechanism that lie between the hydrolytic reaction and product dissociation: these processes cannot be rate-limiting, since k_f would then be smaller than k_a and, to judge from the simulations, have rate

constants 5–10 times higher than the slowest step. We show below that the slow step limiting both k_a and k_f cannot be either the association of the enzyme with its recognition site or the chemical hydrolysis of the DNA.

Hence we propose the following series of enzyme–DNA intermediates for the mode 1 reaction after mixing *Eco*RI restriction endonuclease with plasmid pMB9 [eqn. (3a) for the cleavage of the first strand, and eqn. (3b) for the second, are not full reaction mechanisms since they lack Mg^{2+}]:



The two strands of the DNA at the *Eco*RI recognition site on the covalently closed substrate are shown by S——S, on the open-circle DNA nicked in one strand by P——S and on the linear product cleaved in both strands by P——P. The dimeric *Eco*RI enzyme, each subunit noted in E——E, binds rapidly to its recognition site on the DNA. After binding, we suggest that the active site from each subunit is juxtaposed against one of the scissile phosphodiesters from each strand of the DNA, but that, before either strand can be hydrolysed, the protein subunit at that strand must change its conformation. Presumably, the conformation change would be elicited by specific interactions between the protein and nucleotides within the *Eco*RI recognition site, though flanking sequences may also affect the rate (see Halford *et al.*, 1980). The perturbed circular dichroism of the *Eco*RI enzyme bound to oligonucleotides has also been interpreted as a protein conformation change (Goppelt *et al.*, 1980). Our kinetic data are consistent with the change in protein conformation from E to E* being the slowest steps of both pathways in eqn. (3). Since the two subunits of the *Eco*RI restriction enzyme do not appear to interact co-operatively, the conformation change could occur in either one of the subunits independently of the other. This mechanism automatically explains the dissociation of the enzyme from the DNA between the cleavages of the two strands: if the change in the conformation of one subunit is the slowest step, then the probability of both subunits reaching the catalytic E* state, during the time that the enzyme remains bound to the DNA for one turnover, will be close to zero.

The mode 1 reaction between the *Eco*RI restriction endonuclease and plasmid pMB9 was studied by both gel and fluorescence assays, as in Fig. 1, over a range of both $MgCl_2$ and enzyme concentrations. Increasing concentrations of $MgCl_2$ caused an increase in the reaction rates, with no saturation of the rates at the highest $MgCl_2$ concentrations used (as was also observed in steady-state kinetics; Halford & Johnson, 1981). For example, with the enzyme concentration and other conditions as in Fig. 1, k_f was evaluated as 0.025, 0.10 and 0.23 s^{-1} at 2 mM-, 5 mM- and 15 mM-$MgCl_2$ respectively. In addition, the values of k_a and k_b and the duration of the lag phases underwent parallel changes with the $MgCl_2$ concentration. Hence the relationships between k_a, k_b and k_f, described above as 15 mM-$MgCl_2$, were reproduced at all concentrations of $MgCl_2$ tested (see Table 1 below). Eqn. (3) lacks Mg^{2+} and thus cannot model the effect of Mg^{2+} on rates that we propose to be limited by protein conformation changes. Our data are consistent with these conformation changes being coupled kinetically to the binding of Mg^{2+}.

At fixed concentrations of $MgCl_2$ (either 5 or 15 mM), the apparent rate constants of mode 1 reactions also increased with increasing concentrations of *Eco*RI enzyme, but only up to a saturation value. Parallel changes were observed in k_f, k_a and k_b so that their interrelationship remained as that described above at 17 nM-*Eco*RI enzyme. The variation of the rate constants with the enzyme concentration appeared to lie within experimental error of a rectangular hyperbola: the concentration-dependency is that expected for the apparent rate constants of product formation from a reaction where $[E_0] \gg [S_0]$ but in which $[E_0]$ is not very much greater than K_D and is not shown. The enzyme concentration in Fig. 3 is close to that required for saturation: increasing the concentration of enzyme from 17 nM to 26 and 34 nM resulted in k_f changing from 0.23 s^{-1} to 0.26 and 0.27 s^{-1} respectively. For reactions containing enzyme in molar excess of the DNA, the apparent rate constants for the binding to the recognition site will be linearly dependent on the concentration of enzyme. Hence the above data exclude the possibility that the slow process limiting both k_a and k_f is the binding step. [This applies regardless of whether the *Eco*RI restriction enzyme binds directly to its recognition site or first binds randomly to the DNA and then migrates to the recognition site (Berg *et al.*, 1981).]

Since a lag is not observed before the cleavage of the first strand of the DNA (the gel assay of

covalently closed DNA in Fig. 3), the binding of the *Eco*RI restriction endonuclease to its recognition site on plasmid pMB9 must be almost complete within 1 s. This establishes a lower limit of $1 \times 10^8 M^{-1} \cdot s^{-1}$ for the bimolecular rate constant. The lower limit is consistent with the value of $3.5 \times 10^7 M^{-1} \cdot s^{-1}$ for $k_{cat.}/K_m$ under the same experimental conditions as Fig. 3 (Halford & Johnson, 1981): the variation of $k_{cat.}/K_m$ with salt concentration and pH suggest that it is lower than, and not equal to, the bimolecular rate constant (Halford *et al.*, 1980). However, the lower limit is close to the upper limit that Berg *et al.* (1981) calculated for the diffusion-controlled reaction between a protein and a specific site on a DNA macromolecule. Hence the possibility exists that, at the start of the reaction, the *Eco*RI restriction enzyme binds to its recognition site on the plasmid at a rate that is faster than the diffusion-controlled limit. Other proteins, such as the *lac* repressor, bind to specific sites on DNA with rate constants larger than can be expected from three-dimensional diffusion, but this may be due to facilitated diffusion along the DNA after random binding to the DNA (Berg *et al.*, 1981). Jack *et al.* (1982) presented data on the *Eco*RI restriction enzyme that are consistent with the theory of Berg *et al.* (1981): they proposed that the enzyme dissociates from the intact recognition site in the absence of Mg^{2+} by first transferring to alternative sites on the DNA, after which it leaves the non-specific DNA with a rate constant of about $0.03 s^{-1}$. However, the turnover number of the *Eco*RI restriction enzyme is $0.13 s^{-1}$ (at 20 mM-$MgCl_2$; Halford & Johnson, 1981). Thus, at the end of the catalytic reaction, the enzyme cannot dissociate from the cleaved recognition site by a pathway involving the rate constants from Jack *et al.* (1982). The dissociation of the enzyme from non-specific DNA should occur at the same rate regardless of whether the *Eco*RI recognition site is intact or cleaved. The accelerated dissociation of the *Eco*RI restriction enzyme from DNA, after the catalytic reaction, compared with that calculated by Jack *et al.* (1982) from non-specific DNA could be due to Mg^{2+} enhancing the dissociation rate (see below).

From all of our gel assays on mode 1 reactions, the k_a/k_b ratio was evaluated as 2.2 ± 0.6. This ratio was determined on 42 reactions of the type in Fig. 1(*a*), all employing the same solution conditions as Fig. 1 but covering a range of concentrations for both enzyme (3.4–45 nM) and $MgCl_2$ (2–15 mM). Systematic variations in the k_a/k_b ratio were not observed as a function of either enzyme or $MgCl_2$ concentrations. The scheme in eqn. (3) accommodates the observed ratio. When the dimeric enzyme interacts with the intact substrate, S——S in eqn. (3a), either of the two subunits in E——E may undergo the conformation change to E* before cleaving one strand. In contrast, when the enzyme interacts with the nicked DNA, P——S in eqn. (3b), the only route to a productive complex is for the conformation change to occur in the one subunit whose active site is on the intact strand; a conformation changes in the subunit next to the P strand of P——S generates an abortive complex. Thus, if each conformation change that could lead to DNA cleavage occurs with the same rate constant, that in eqn. (3a) will appear to be twice as fast as that in eqn. (3b). Since our reactions contain ethidium bromide, the deviation in the observed ratio of k_a/k_b from the statistical expectation of 2.0 may be accounted for by additional ethidium binding to the DNA before the second strand has been cleaved and thus slightly inhibiting the latter (Halford & Johnson, 1981).

Reactions of enzyme with DNA

We have also examined mode 2 reactions, in which the pre-equilibrium of *Eco*RI restriction endonuclease and plasmid pMB9 will already contain enzyme bound to the recognition site on the DNA before the initiation of the reaction by adding $MgCl_2$. Product formation was measured on parallel reactions by either gel or fluorescence assays (Fig. 4). The reactions were studied over ranges of both enzyme and $MgCl_2$ concentrations but keeping enzyme in excess of DNA. With mode 2 mixing, variations in the enzyme concentration above 10 nM did not alter the kinetics of product formation on either assay. At lower concentrations of enyzme, biphasic kinetics were observed: one portion of the reaction amplitude proceeded at the rates characteristic of the mode 2 reaction at above 10 nM-enzyme concentration, and the remainder of the amplitude yielded rate constants that were equal to those from the mode 1 reaction of the same final composition. This behaviour is as expected for mode 2 reactions in which the enzyme concentrations are not much larger than the K_D for the recognition site: in such a pre-equilibrium of enzyme and DNA, only a portion of the *Eco*RI recognition sites would be bound to the enzyme and be cleaved as in the mode 2 reaction. All of the mode 2 reactions described below contain enzyme concentrations above 10 nM. In contrast with mode 1 reactions, where the reactions profiles at different $MgCl_2$ concentrations remained qualitatively similar to that in Fig. 3, the kinetics of mode 2 reactions were quantitatively different at low concentrations of $MgCl_2$ ($\leqslant$5 mM; Fig. 4*a*) compared with those at high concentrations of $MgCl_2$ ($\geqslant$15 mM; Fig. 4*b*).

For one mode 2 reaction at a low $MgCl_2$ concentration, Fig. 4(*a*) shows the time courses for the cleavage of the *Eco*RI recognition site at the first and at the second strands of the DNA (from the respective concentrations of covalently closed DNA

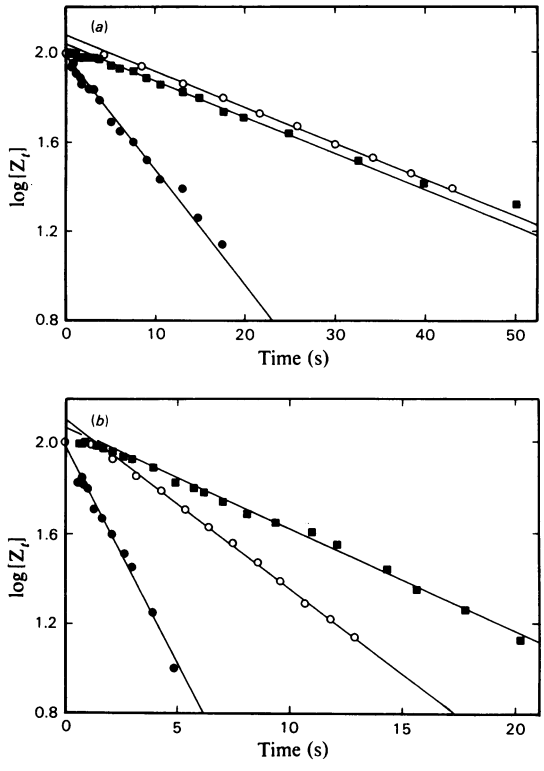


Fig. 4. *Reaction of enzyme–DNA against two concentrations of $MgCl_2$*

Reaction mixtures contained *Eco*RI restriction endonuclease (17 nM), plasmid pMB9 (1.5 nM in gel assays, 0.20 nM in fluorescence assays) and $MgCl_2$ (2.0 mM in *a*, 15 mM in *b*) in 50 mM-Tes buffer, pH 7.0, containing 50 mM-NaCl, 10 μM-ethidium bromide and 0.01% gelatin, at 22°C. Before the mixing, enzyme and DNA were in one solution, $MgCl_2$ was in the second and all other components were in both. The reactions were monitored by both gel and fluorescence assays (not shown), and the data are given in semi-logarithmic form. At time t, $[Z_t]$ is the concentration of either covalently closed DNA (●) or linear DNA (■) from gel assays or is proportional to the fluorescence enhancement (○): all three have been normalized on a scale from $[Z_0] = 100$ to $[Z_\infty] = 0$. The lines drawn are single exponential fits to all data within the following time spans: in (*a*) at 2 mM-$MgCl_2$, covalently closed DNA from 0 to 10.4 s ($k_a = 0.12\,s^{-1}$), linear DNA from 8 to 42 s ($k_b = 0.035\,s^{-1}$), fluorescence from 5.1 to 40 s ($k_f = 0.037\,s^{-1}$); in (*b*) at 15 mM-$MgCl_2$, covalently closed DNA from 0 to 4.9 s ($k_a = 0.45\,s^{-1}$), linear DNA from 6.2 to 20.2 s ($k_b = 0.10\,s^{-1}$), fluorescence from 4.3 to 11.8 s ($k_f = 0.17\,s^{-1}$).

and linear DNA on the gel assay) and also the fluorescence signal. The kinetic parameters from this reaction are compared in Table 1 with those from the mode 1 reaction of identical composition. The

Table 1. *Comparison of mixing procedures used to generate reaction mixtures*

The mode 1 and the mode 2 mixing procedures were used to generate reaction mixtures containing *Eco*RI restriction endonuclease (17 nM), plasmid pMB9 (1.5 nM for gels, 0.20 nM for fluorescence) and $MgCl_2$ (2.0 mM) in 50 mM-Tes buffer, pH 7.0, containing 50 mM-NaCl, 10 μM-ethidium bromide and 0.01% gelatin, at 22°C. For both, product formation was monitored by gel assays (to determine k_a and k_b) and by fluorescence assays (to yield k_f). The apparent rate constants are average values from at least three reactions of each type, one of which is shown in Fig. 4(*a*).

	Apparent rate constants (s^{-1})	
	Mode 1	Mode 2
k_a	0.046	0.13
k_b	0.021	0.037
k_f	0.052	0.042

alteration to the mixing procedure between modes 1 and 2 caused three major differences to the kinetics. Firstly, the value obtained for k_a, the apparent rate constant for the cleavage of the first strand of the DNA, was larger in the mode 2 reaction than in mode 1. [The chemical hydrolysis of the phosphodiester at the active site of the *Eco*RI endonuclease will, most probably, occur at the same rate regardless of the procedure used to mix the reagents. Hence the higher value of k_a in the mode 2 reaction indicates that the chemical hydrolysis of the DNA cannot be the slow step in eqn. (3a) whose rate determines both k_a and k_f in the mode 1 reaction.] Secondly, the value of k_b for the cleavage of the second strand was also higher after mode 2 mixing, but the increase from the equivalent mode 1 reaction was less than that for k_a: hence the k_a/k_b ratio is different. From 14 gel assays on mode 2 reactions at low concentrations of $MgCl_2$, as in Fig. 4(*a*) but covering the range 1–5 mM-$MgCl_2$, the values of both k_a and k_b increased with increasing concentrations of $MgCl_2$ but the k_a/k_b ratio remained constant at 3.3 ± 0.5.

The third difference in the kinetics between mode 1 and mode 2 reactions at low $MgCl_2$ concentrations was that the fluorescence records in the mode 2 reactions were virtually superimposable, both in their lag and exponential phases, upon the time courses for the cleavage of the second strand (Fig. 4*a*). This observation differs from mode 1 reactions (Fig. 3 and Table 1), where the exponential phases of the fluorescence records yielded apparent rate constants (k_f) that were equal to those for cutting the first strand (k_a) rather than the second (k_b). If the fluorescence is enhanced only when the DNA, cleaved in at least one strand, dissociates from the enzyme, then the data in Fig. 4(*a*)

demonstrate that both strands of the DNA must be cleaved before the enzyme dissociates from the DNA at the end of the mode 2 reaction. This constrasts with the mode 1 reaction, where the enzyme appears to dissociate from the DNA between the two cleavage reactions.

The scheme in eqn. (3), used above to describe the reaction after mixing *Eco*RI restriction endonuclease and plasmid pMB9 (mode 1), is adapted in eqn. (4) in order to account for the altered kinetics when the reaction is initiated by the addition of a low concentration of $MgCl_2$ to the preformed mixture of enzyme and DNA (mode 2):

$$\begin{matrix} E^*\!\!-\!\!-E^* \\ \dot{S}\!\!-\!\!-\dot{S} \end{matrix} \longrightarrow \begin{matrix} E^*\!\!-\!\!-E^* \\ \dot{P}\!\!-\!\!-\dot{S} \end{matrix} \longrightarrow \begin{matrix} E^*\!\!-\!\!-E^* \\ \dot{P}\!\!-\!\!-\dot{P} \end{matrix} \longrightarrow \begin{matrix} E\!\!-\!\!-E \\ + \\ P\!\!-\!\!-P \end{matrix} \qquad (4)$$

In the absence of $MgCl_2$, the *Eco*RI enzyme will bind to its recognition site on the DNA (Halford & Johnson, 1980). During the interval between the preparation of the mixture of enzyme and DNA and the initiation of the reaction with $MgCl_2$ (typically several minutes in our experiments), we suggest that sufficient time will elapse to allow both subunits of the *Eco*RI dimer to reach the active E* conformation. Both strands of the DNA can then be cleaved during the lifetime of a single enzyme–DNA complex. Hence, on our model, the mode 2 reaction by-passes the slow conformation change that, in the mode 1 reaction, follows the binding of the enzyme to its recognition site and then limits the enzyme to one DNA cleavage per enzyme–DNA complex.

In the mode 2 reaction, the actual rate constants for the first two steps in eqn. (4) are not given by the apparent rate constants, k_a and k_b, for cutting each of the two strands, and instead will be much larger: eqn. (4) lacks Mg^{2+} and the values of both k_a and k_b increase with increasing $MgCl_2$ from 1 to 5 mM. If the equilibration of Mg^{2+} on to the enzyme–DNA complex is rapid, then for the mechanism

$$E \cdot S + Mg \overset{K_c}{\rightleftharpoons} E \cdot S \cdot Mg \xrightarrow{k_2} E \cdot P \cdot Mg \qquad (5)$$

where K_c is the equilibrium dissociation constant for the Mg^{2+} cation, the apparent rate constant for product formation ($k_{obs.}$) is given by:

$$k_{obs.} = \frac{k_2}{1 + \frac{K_c}{[Mg]}} \qquad (6)$$

Hence, when $K_c \gg [Mg]$, $k_{obs.}$ will be linearly dependent on the ratio $[Mg]/K_c$. We assume that, when the *Eco*RI restriction enzyme is bound to its recognition site on the DNA, both of its two subunits possess the same intrinsic affinity for Mg^{2+}; the first ion of Mg^{2+} will then bind to either subunit with a dissociation constant one-half of K_c, but the second ion of Mg^{2+} will bind to the enzyme (already containing one Mg^{2+} ion) with an equilibrium constant of twice K_c (Tanford, 1961). Thus, when the apparent rate constants for the cleavage of each DNA strand are dependent on the fractional saturation of each subunit with Mg^{2+}, we would expect a 4-fold difference between the two cleavage rates. The observed ratio of 3.3 for k_a/k_b from mode 2 reactions at low $MgCl_2$ concentrations approaches the statistical expectation.

When mode 2 reactions were performed by adding a high concentration of $MgCl_2$ (at least 15 mM) to the complex between the *Eco*RI restriction enzyme and the DNA (Fig. 4*b*), the reaction profiles differed quantitatively from those at low $MgCl_2$ concentrations (Fig. 4*a*). At the higher concentration (Fig. 4*b*), the apparent rate constant for cutting the first strand (k_a) was again faster than the equivalent mode 1 reaction (Fig. 3): this cleavage had a half-time of 2 s, too fast for our gel assays to yield reliable values. However, the fluorescence signal from these reactions displayed, after a short lag, an exponential phase in which the value of k_f differed beyond experimental error from either k_a or k_b and was between these. To account for the dissociation of the enzyme from the DNA before the cleavage of the second strand in mode 2 reactions at high (but not at low) $MgCl_2$ concentrations, perhaps a rate constant for the dissociation of the enzyme from the DNA (at some stage of the reaction) is larger when at least one ion of Mg^{2+} is bound than in its absence. Hence, at increasing concentrations of $MgCl_2$, there could be a higher probability of dissociation before completion of the reaction: the apparent value of k_f would reflect this probability and thus be lower than the actual dissociation rate constant. This suggestion is compatible with two previous observations: firstly, the K_D for the binding of the enzyme to non-specific DNA is increased by $MgCl_2$ (Halford & Johnson, 1980); secondly, the K_m for its recognition site on plasmid pMB9 is also increased by increasing concentrations of $MgCl_2$ (Halford & Johnson, 1981). Moreover, the apparent rate constant for the cleavage of the second strand (k_b) during the mode 2 reaction in Fig. 4(*b*) has the same value, within experimental error, as that for k_b in the equivalent mode 1 reaction (Fig. 3); this is also consistent with dissociation before the cutting of the second strand.

Modrich & Zabel (1976) carried out a single turnover of the *Eco*RI restriction endonuclease by adding $MgCl_2$ to the pre-formed complex of the

enzyme and plasmid DNA (as in our mode 2 reactions), measured the rate of formation of linear DNA, which they presumed to remain enzyme-bound, and, since this rate was faster than $k_{cat.}$ from the steady state, they concluded that product dissociation was rate-limiting. However, the comparison used by Modrich & Zabel (1976) is invalid. The only valid comparison would have been between the mode 1 single turnover and the steady state: each reaction in the steady-state must involve the association of the enzyme with DNA, whereas the mode 2 single turnover by-passes the events coupled to the association. Yet there remains a discrepancy between the steady state and the mode 1 single turnover. A value of $0.1\,s^{-1}$ was obtained for $k_{cat.}$ from steady-state kinetics of the cleavage of the first strand at the *Eco*RI recognition site on plasmid pMB9 (extrapolated to infinite [DNA] at 15mM-$MgCl_2$; Halford & Johnson, 1981); under the same experimental conditions (Fig. 3), the mode 1 single turnover yielded a value of about $0.3\,s^{-1}$ for the cleavage of the first strand (from either k_a or k_f extrapolated to infinite [E]). Two causes may account for this discrepancy, even though product dissociation cannot be rate-limiting (Fig. 3). Firstly, if an enzyme mechanism contains two or more slow steps with similar rate constants (for example, k_x and k_y in eqn. 2), then:

$$k_{cat.} = \frac{k_x \cdot k_y}{k_x + k_y} \qquad (7)$$

so $k_{cat.}$ must have a lower value than either k_x or k_y; but in a single turnover the exponential phase of product formation yields a rate constant that is equal to either k_x or k_y, whichever is the smaller. Secondly, though our preparations of *Eco*RI restriction endonuclease are reasonably homogeneous, we cannot exclude the possibility that a fraction of the protein is inactive: the presence of inactive enzyme would result in $k_{cat.}$ being under-estimated but it would not affect single-turnover kinetics.

We thank Professor H. Gutfreund, Dr. D. W. Yates and Dr. M. A. Geeves (of this department) for advice and the loan of equipment, Professor T. Atkinson and Dr. V. A. Quirk (Centre for Applied Microbiology and Research, Porton, Wilts., U.K.) for help with the enzyme preparations, and the Science and Engineering Research Council for financial support.

References

Bauer, W. (1978) *Annu. Rev. Biophys. Bioeng.* **7**, 287–313

Berg, O. G., Winter, R. B. & Von Hippel, P. H. (1981) *Biochemistry* **20**, 6929–6948

Campbell, V. W. & Jackson, D. A. (1980) *J. Biol. Chem.* **255**, 3726–3735

Capellos, C. & Bielski, B. H. J. (1972) *Kinetic Systems*, pp. 53–58, John Wiley and Sons, New York

Clore, G. M., Gronenborn, A. M. & Davies, R. W. (1982) *J. Mol. Biol.* **155**, 447–466

Fuortes, M. G. F. & Hodgkin, A. L. (1964) *J. Physiol. (London)* **172**, 239–263

Goppelt, M., Pingoud, A., Maass, G., Mayer, H., Kustler, H. & Frank, R. (1980) *Eur. J. Biochem.* **104**, 101–107

Greene, P. J., Gupta, M., Boyer, H. W., Brown, W. E. & Rosenberg, J. M. (1981) *J. Biol. Chem.* **256**, 2143–2153

Halford, S. E. & Johnson, N. P. (1980) *Biochem. J.* **191**, 593–604

Halford, S. E. & Johnson, N. P. (1981) *Biochem. J.* **199**, 767–777

Halford, S. E., Johnson, N. P. & Grinsted, J. (1979) *Biochem. J.* **179**, 353–365

Halford, S. E., Johnson, N. P. & Grinsted, J. (1980) *Biochem. J.* **191**, 581–592

Hedgpeth, J., Goodman, H. M. & Boyer, H. W. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3448–3452

Imber, R. & Bickle, T. A. (1981) *Eur. J. Biochem.* **117**, 395–399

Jack, W. E., Terry, B. J. & Modrich, P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4010–4014

Kelly, T. J. & Smith, H. O. (1970) *J. Mol. Biol.* **51**, 393–409

Langowski, J., Pingoud, A., Goppelt, M. & Maass, G. (1980) *Nucleic Acid Res.* **8**, 4727–4736

Langowski, J., Urbanke, C., Pingoud, A. & Maass, G. (1981) *Nucleic Acids Res.* **9**, 3483–3490

Lu, A. L., Jack, W. E. & Modrich, P. (1981) *J. Biol. Chem.* **256**, 13200–13206

Maxwell, A. & Halford, S. E. (1982) *Biochem. J.* **203**, 85–92

McCalla, T. R. (1967) *Introduction to Numerical Methods and Fortran Programming*, p. 258, John Wiley and Sons, New York

Modrich, P. & Zabel, D. (1976) *J. Biol. Chem.* **251**, 5866–5874

Newman, A. K., Rubin, R. A., Kim, S. H. & Modrich, P. (1981) *J. Biol. Chem.* **256**, 2131–2139

Rodriguez, R. L., Bolivar, F., Goodman, H. M., Boyer, H. W. & Betlach, M. (1976) in *Molecular Mechanisms in the Control of Gene Expression* (Nierlich, D. P., Rutter, W. J. & Fox, C., eds.), pp. 471–477, Academic Press, New York

Roulland-Dussoix, D., Yoshimori, R., Greene, P. J., Betlach, M., Goodman, H. M. & Boyer, H. W. (1974) in *American Society for Microbiology Conference on Bacterial Plasmids* (Schlessinger, D., ed.), pp. 187–198, ASM Press, Washington

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 526–535, John Wiley and Sons, New York