The *EcoRI* restriction endonuclease, covalently closed DNA and ethidium bromide

Stephen E. HALFORD and Nicola P. JOHNSON

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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The reactions of the *EcoRI* restriction endonuclease on the covalently closed DNA of plasmid pMB9 were studied in the presence of ethidium bromide. At the concentrations of ethidium bromide tested, which covered the range over which the DNA is changed from negatively to positively supercoiled, the dye caused no alteration to the rate at which this enzyme cleaved the covalently closed DNA to yield the open-circle form, but the rate at which these open circles were cleaved to the linear product could be inhibited. The fluorescence change, caused by ethidium bromide binding with different stoichiometries to covalently closed and open-circle DNA, provided a direct and sensitive signal for monitoring the cleavage of DNA by this enzyme. This method was used for a steady-state kinetic analysis of the reaction catalysed by the *EcoRI* restriction enzyme. Reaction mechanisms where a complex between DNA and Mg^{2+} is the substrate for this enzyme were eliminated, and instead DNA and Mg^{2+} must bind to the enzyme in separate stages. The requisite controls for this fluorimetric assay in both steady-state and transient kinetics studies, and its application to other enzymes that alter the structure of covalently closed DNA, are described.

Class-II restriction endonucleases recognize specific nucleotide sequences in DNA and, in the presence of Mg^{2+} , cleave both strands of the duplex DNA at fixed locations relative to their recognition sites. Several methods have been employed to monitor the reaction of these enzymes on DNA substrates. In one widely used technique, the reaction between the restriction enzyme and the DNA is terminated after a given time by the addition of EDTA and the products are then separated from the substrate by gel electrophoresis: the DNA is located on the gel by staining with ethidium bromide (Sharp *et al.*, 1973). Quantitative data about the concentrations of substrate and product in the gel can be obtained by using radiolabelled DNA (Greene *et al.*, 1975; Halford *et al.*, 1979), by fluorescence measurements (Forsblom *et al.*, 1976) or by photographic analysis (Woodhead & Malcolm, 1980). Linked assay systems have also been described for restriction endonucleases (Modrich & Zabel, 1976). But all of these methods suffer the major disadvantages of being laborious and of yielding discontinuous records of product formation. Each sample subjected to electrophoresis or

used in a linked assay corresponds to a single time point in the reaction, and a large number of these must be analysed in order to establish the kinetics of each reaction.

However, ethidium bromide is intensely fluorogenic when bound to duplex DNA, with the same quantum yield whether bound to covalently closed or open-circle DNA (Le Pecq, 1971). DNA duplexes possess one of three structures: covalently closed DNA, in which both strands are circular; open-circle DNA, in which one strand is circular while the other is lacking a phosphodiester bond; linear DNA, in which the termini of both strands are free. The latter two are not topologically constrained and thus bind ethidium bromide to the same extent, but covalently closed DNA binds this dye with a different stoichiometry (Bauer & Vinograd, 1968). Hence the fluorescence signal caused by a change in the amount of ethidium bromide bound to the DNA has been used to assay a number of enzymes that alter the structure of circular DNA molecules (Morgan *et al.*, 1979). But the majority of such assays have still yielded discontinuous records of product formation: the optimal difference between covalently closed and other forms of DNA, with regard to fluorescence from bound ethidium bromide, is obtained by taking samples at timed

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

intervals from the reaction mixture of enzyme and DNA, heating to 95°C and then adding ethidium bromide at pH 12 (Morgan *et al.*, 1979). These conditions are incompatible with enzyme activity.

Therefore we report here on the reactions of the *EcoRI* restriction endonuclease with a covalently closed DNA substrate in the presence of ethidium bromide. If this enzyme were to cleave DNA at the same rate in the presence of ethidium bromide as in its absence, and if the rate of ethidium bromide binding to DNA was fast compared with the enzyme reaction, then it would be possible to obtain a continuous record of the reaction by directly observing the fluorescence change from a solution containing enzyme, covalently closed DNA and ethidium bromide. The *EcoRI* restriction endonuclease is a class-II enzyme which interacts with the symmetrical base sequence, 5'-G-A-A-T-T-C-3', in DNA and cleaves both strands of the duplex between the first and second nucleotides from the 5'-end of the recognition sequence (Hedgpeth *et al.*, 1972). The enzyme is very specific for its recognition sequence and cleaves it more than 50 million times faster than it does alternative sequences on DNA (Halford & Johnson, 1980).

Materials and methods

Fluorescence measurements at equilibrium and over long time periods (>30s) were made on 600 µl samples, thermostatically maintained at 22°C, in the 5 mm cell of a Perkin Elmer 3000 spectrofluorimeter equipped with a Bryans 28000 y/t recorder. Excitation of ethidium bromide-DNA complexes was at 335 nm and the emission observed at 595 nm; 10 nm slits were employed on both monochromators. Fluorescence measurements over short time periods (<30s) were made at room temperature in the stopped-flow fluorimeter of Dr. D. W. Yates (Bagshaw *et al.*, 1972) with the excitation beam being passed through both the monochromator at 335 nm with 10 nm slits and a strip interference filter (type UMS, from Barr and Stroud, Glasgow, Scotland, U.K.) set to this wavelength; emission was observed through a cut-off filter (type KV 550, from Schott-Jena, Mainz, West Germany). The stopped-flow fluorimeter was used as described by Halford (1975), except that a different procedure, obtained from Professor H. Gutfreund, was used for data capture and analysis. The amplified output from the emission photomultiplier, after correction for variations in the excitation beam, was recorded (via a Datalab DL 905 transient recorder) on cassette tapes in a Racal deck controlled by an Intel 8080 microprocessor. The experimental records stored on the cassettes, each consisting of 1000 data points of fluorescence readings against time, were subsequently transferred to a PDP 8/E

computer for averaging and for non-linear least-squares fitting procedures.

The *EcoRI* restriction endonuclease was purified to over 90% homogeneity as described previously (Halford *et al.*, 1980). The molarities of solutions of *EcoRI* restriction enzyme (and hence catalytic centre activities) are given in terms of the dimeric protein of mol.wt. 62000. The *SalGI* restriction endonuclease, purified to over 80% homogeneity, was a gift from Mr. A. Maxwell (this laboratory).

The plasmid pMB9 (Rodriguez *et al.*, 1976) was obtained from a transformant of *Escherichia coli* strain UB 5852 (*rec A-56, thy, pro, met, nalA*) that contained the monomeric form of plasmid pMB9, and which has maintained over 4 years the plasmid in its monomeric state. Growth of the bacterial cultures (except that now 50 mg of chloramphenicol was added per litre of culture in late exponential phase) and the initial isolation of the plasmid DNA followed the procedure of Grinstead *et al.* (1978). The plasmid was purified by two centrifugations to equilibrium, both for 16 h at 50000 rev./min, at 15°C, in the TV 865B rotor of the Sorvall OTD-50-B centrifuge; the sample volume used in both centrifugations was 16 ml, adjustments to the components listed below being made with 50 mM-Tris/50 mM-EDTA, pH 7.5, to which had been added 1.075 g of CsCl/ml. For the first centrifugation, 1.075 g of CsCl was added per ml of DNA solution, and this was added to 20 µl of ethidium bromide in water (3 mg/ml). This centrifugation yielded a single band of DNA, visible under u.v. illumination, that contained both covalently closed and other forms of DNA. The solution containing the band of DNA from the first gradient was collected by syringe, mixed with 2.0 ml of ethidium bromide in water (3 mg/ml) and subjected to a second centrifugation as above. After the second centrifugation, two bands of DNA were observed and the lower, containing the covalently closed DNA, was collected. [The conditions for the second centrifugation differ from the first in both ethidium bromide and CsCl concentrations and, since the buoyant density of DNA varies with ethidium bromide concentration (Bauer & Vinograd, 1968), cellular material that otherwise contaminates the band of covalently closed DNA on the second gradient is removed previously by the first.] The solution of covalently closed DNA was washed extensively with CsCl-saturated propan-2-ol, dialysed against 10 mM-Tris/0.1 mM-EDTA, pH 7.5, and the DNA stored at 4°C in this solution over chloroform.

Plasmid pMB9 labelled with [³H]thymidine was prepared as described above, except that 1 litre of bacterial growth media also contained 125 mg of 2'-deoxyadenosine and that 2.5 mCi of [methyl-³H]-thymidine (The Radiochemical Centre, Amersham)

was added with the chloramphenicol. In electrophoreses of plasmid pMB9 directly after preparation, more than 90% of the DNA had the mobility characteristic of the covalently closed form of the monomeric plasmid, but over a period of weeks the proportion of covalently closed DNA declined and that of the open-circle form increased. All of the experiments with plasmid pMB9 described here were carried out with DNA preparations containing more than 80% covalently closed DNA, each preparation being used for no longer than 3 weeks before being discarded. (Neither the amplitudes nor the rates of fluorescence change reported below have been corrected for the small amount of open-circle DNA present in each reaction at zero time.) The linear form of plasmid pMB9 was obtained by digestion with the *EcoRI* restriction endonuclease. The molarities of solutions of plasmid pMB9 are given in terms of DNA molecules of mol.wt. 3.6×10^6 : this is equal to the molarities of *EcoRI* recognition sites (Rodriguez *et al.*, 1976) if both strands of the DNA duplex are considered to constitute one recognition site.

Ethidium bromide (Boehringer) solutions were kept in the dark; their concentrations were determined by absorption at 480nm, with $\epsilon = 5450 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Le Pecq, 1971). All other materials and methods were described by Halford *et al.* (1979, 1980).

Results and discussion

Binding of ethidium bromide to DNA

The fluorescence emission from solutions containing either covalently closed or linear DNA from plasmid pMB9 was measured over a range of ethidium bromide concentrations (Fig. 1). These fluorescence data are equivalent to previous studies on the sedimentation properties of covalently closed and open-circle DNA at different ethidium bromide concentrations (Crawford & Waring, 1967; Bauer & Vinograd, 1968) and can be accommodated by the same mechanism. When isolated from *E. coli*, DNA from plasmid pMB9 contains negative supercoils (Bauer, 1978), which become relaxed by binding low amounts of ethidium bromide and which are converted into positive supercoils after binding larger amounts of ethidium bromide. The conversion of a negatively supercoiled DNA molecule into a relaxed structure is exergonic, whereas the introduction of positive supercoils into the relaxed molecule is endergonic (Bauer, 1978). Thus at the start of the titrations in Fig. 1, at low concentrations of ethidium bromide, more dye is bound to the covalently closed DNA than to the linear DNA, so that the fluorescence from the covalently closed DNA is higher. But with high concentrations of ethidium bromide at the end of the titration, less dye

Fig. 1. *Ethidium bromide titrations*

The fluorescence emission was recorded from solutions containing the concentrations of ethidium bromide noted above and 50mM-Tes, 50mM-NaCl, 10mM-MgCl₂, 0.01% gelatin, pH 7.0, and 0.56 nM-DNA from plasmid pMB9 (either covalently closed or linear). The values for the fluorescence difference (ΔF) were calculated at each ethidium bromide concentration from the readings with linear DNA (F_l) minus those with covalently closed DNA (F_c) and are given as ratios of $\Delta F/F_c$. The values of F_c and F_l have been corrected for the fluorescence of free ethidium bromide.

is bound to the covalently closed than to the linear DNA, so that the linear DNA then yields the larger fluorescence.

Titration similar to those in Fig. 1 were carried out under different conditions. The fluorescence difference between covalently closed and linear DNA at above 10 μM-ethidium bromide did not vary significantly with changes to either the NaCl or MgCl₂ concentrations. But the concentrations of ethidium bromide required to reach the cross-over point, where the fluorescences from the covalently closed and linear DNA samples were equal, depended on both the NaCl and MgCl₂ concentrations: thus both the sign and amplitude of the fluorescence difference at below 3 μM-ethidium bromide could be altered by changes to the solution composition. The cross-over point will occur at a concentration of ethidium bromide at which the covalently closed DNA has been unwound to its relaxed configuration; duplex winding is affected by NaCl and MgCl₂ (Bauer, 1978).

Titration were also carried out at fixed concentrations of ethidium bromide with varied DNA concentrations (Fig. 2). Over the range shown, the

fluorescence increased linearly with increasing DNA concentrations, for both covalently closed and linear DNA. But at high DNA concentrations, above 4 nM-plasmid pMB9 (results not shown), the increments in fluorescence with increasing DNA concentrations declined from linearity. The plasmid pMB9 contains about 5500 base-pairs (Rodriguez *et al.*, 1976) so that, at saturation with ethidium bromide, each covalently closed molecule of this DNA will bind about 1100 molecules of the dye, whereas each linear molecule will take up about 2200 dye molecules; the stoichiometries are from Bauer & Vinograd (1968). Thus the range of DNA concentrations that yield fluorescence signals linearly proportional to DNA is limited to where the concentration of binding sites for ethidium bromide is smaller than the total concentration of the dye.

Fig. 2. DNA titrations

The fluorescence emission was recorded from solutions containing the concentrations of DNA from plasmid pMB9 shown and 50 mM-Tes, 50 mM-NaCl, 10 mM-MgCl₂, 10 μ M-ethidium bromide and 0.01% gelatin, pH 7.0. The DNA was either covalently closed (●) or linear (○).

The addition of *Eco*RI restriction endonuclease up to 75 nM caused no alteration in the fluorescence emission from either 0.5 μ M- or 10 μ M-ethidium bromide. Since the emission is sensitive to the environment of the ethidium bromide (Le Pecq, 1971), it is unlikely that the dye binds extensively to the protein. But the addition of *Eco*RI restriction endonuclease to solutions containing 10 μ M-ethidium bromide and 0.56 nM-DNA (either covalently closed or linear plasmid pMB9) in 50 mM-Tes/50 mM-NaCl/10 mM-EDTA/0.01% gelatin, pH 7.0, decreased the fluorescence signal; with EDTA present, the DNA is not cleaved. The reduction was negligible at enzyme concentrations below 25 nM, but at 75 nM-enzyme the fluorescence enhancement from adding the DNA to the ethidium bromide was 25% of that observed in the absence of the enzyme. Since the *Eco*RI restriction enzyme can bind to DNA lacking the *Eco*RI recognition sequence (Halford & Johnson, 1980), this effect probably arises from competition between the enzyme and the dye for binding sites at all DNA sequences, with the binding of both enzyme and dye at the same site being incompatible.

The stopped-flow fluorimeter was used to measure the kinetics of ethidium bromide binding to DNA, under conditions used below in studies on the *Eco*RI-catalysed cleavage of DNA. At both concentrations of ethidium bromide tested, and with both the covalently closed and linear forms of plasmid pMB9, the fluorescence enhancements after the mixing of ethidium bromide and DNA could be described by single exponentials. Apparent first-order rate constants are given in Table 1. For the binding to linear DNA, the apparent rate constants are consistent with the values of $8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 35 s^{-1} for the association and dissociation rate constants obtained by Jovin (1975) in fluorescence temperature-jump studies on ethidium bromide binding to poly(dA-dT). Though we have not studied in detail the mechanism of dye binding to covalently

Table 1. Kinetics of ethidium bromide binding to DNA

Apparent first-order rate constants (k_0) were evaluated from stopped-flow records of the fluorescence enhancements after the mixing of ethidium bromide and DNA solutions to yield reactions containing the concentrations of ethidium bromide given below. 0.28 nM-DNA from plasmid pMB9 (either covalently closed or linear as indicated), 50 mM-Tes, 50 mM-NaCl, 5 mM-MgCl₂ and 0.01% gelatin, pH 7.0. At each ethidium bromide concentration, the same amplification on the emission photomultiplier was used for the reactions with both forms of DNA: amplitudes of the reactions were measured and the ratio of that with covalently closed DNA to that with linear DNA is reported here.

[Ethidium bromide] (μ M)	k_0 (s^{-1}) with:		Ratio of amplitudes (covalently closed/ linear DNA)
	Covalently closed DNA	Linear DNA	
0.5	48	47	1.35
10	104	156	0.59

closed DNA, it is probable that its lower apparent rate constant at $10\mu\text{M}$ -ethidium bromide results from a decreased association rate constant with an unaltered dissociation rate constant. But all of the apparent rate constants in Table 1 are much higher than the catalytic centre activity of the *EcoRI* restriction endonuclease on plasmid pMB9 (see Fig. 5 below).

Two experiments showed that the covalently closed form of plasmid pMB9 could be driven through the mixing jets of the stopped-flow apparatus without shear damage. Firstly, the relative amplitudes of the fluorescence enhancements after the mixing of ethidium bromide and DNA are given in Table 1. At $0.5\mu\text{M}$ -ethidium bromide, the fluorescence amplitude was larger with the covalently closed DNA than with the linear DNA, whereas the converse held at $10\mu\text{M}$ -ethidium bromide. These observations concur with the fluorescence titrations in Fig. 1. If the covalently closed DNA had been sheared by the stopped-flow fluorimeter, the fluorescence amplitude from its binding of ethidium bromide would have been identical at all dye concentrations with that from linear DNA. Secondly, a sample of [^3H]DNA from plasmid pMB9 in its covalently closed form was passed through the stopped-flow machine and then analysed by agarose-gel electrophoresis (Halford *et al.*, 1979). In both this sample of the DNA, and another sample from the same preparation that had not been passed through the machine, 83% of the total DNA was found to be in its covalently closed form.

Cleavage of DNA-ethidium bromide complex

The monomeric form of plasmid pMB9 carries one *EcoRI* recognition site (Rodriguez *et al.*, 1976). The cleavage of this plasmid by the *EcoRI* restriction endonuclease involves two separate reactions:

(Halford *et al.*, 1979). In the first reaction, the covalently closed DNA (A) is cleaved in one strand of the duplex to yield the open-circle DNA (B). In the second, the intact strand of the open-circle DNA is cleaved at the *EcoRI* recognition site to yield the linear DNA (C). For an enzyme-catalysed reaction where the initial concentrations of both enzyme and substrate are below the K_m value, the time course for the decrease in the substrate concentration (and the increase in the product concentration) is exponential. For the *EcoRI*-catalysed cleavage of plasmid pMB9, two apparent first-order rate constants (k_a and k_b) are required to describe the reaction in eqn. (1). But since the concentrations of all three forms of the DNA can be determined after gel electrophoresis of samples withdrawn at timed intervals from the reaction, both k_a and k_b may be determined (Halford *et al.*, 1979).

We have studied the reaction of the *EcoRI* restriction enzyme on plasmid pMB9 by gel assays in both the absence and presence of ethidium bromide (Table 2). In the absence of ethidium bromide, the ratio between the two apparent rate constants (k_a/k_b) was found to be 1.6, approximately equal to the statistical factor of 2:1 (Halford *et al.*, 1979); the intact DNA substrate (A) contains two strands, either of which may be cleaved to yield the first product (B), whereas the latter DNA contains only one strand that can be cleaved to yield the final product (C). Hence the enzyme shows equal reactivities for single-strand cleavage on either substrate and is free to dissociate from the DNA after catalysing the first reaction. The addition of ethidium bromide caused no alteration to the values of k_a . However the values of k_b could be decreased

Table 2. *Gel and fluorescence assays*

Reactions at 22°C contained, in a volume of $600\mu\text{l}$, 0.30 nM -*EcoRI* restriction endonuclease, 2.8 nM -[^3H]DNA from plasmid pMB9 (initially 84% covalently closed), 50 mM -Tes, 50 mM -NaCl, 10 mM - MgCl_2 , 0.01% gelatin, pH 7.0 and ethidium bromide as given below. For each gel assay, 15 samples each of $30\mu\text{l}$ were removed at timed intervals after the addition of enzyme, added immediately to $20\mu\text{l}$ of 0.1 M -EDTA/ 0.1 M -Tris/ 40% (w/v) sucrose/ 0.02% Bromophenol Blue, pH 8.0, and each was loaded into one slot of an agarose slab gel: a zero time point was taken before the addition of enzyme and an end point 10 min after adding $0.3\mu\text{g}$ of *EcoRI* endonuclease to the residual reaction mixture left at the penultimate time point: the conditions for gel electrophoresis, the determination of the concentrations of covalently closed, open-circle and linear DNA at each time point and hence the evaluation of k_a and k_b (eqn. 1) were all described by Halford *et al.* (1979). For each fluorescence assay, the reaction mixture without enzyme was placed in the spectrofluorimeter, the signal for full-scale deflection on the y/t recorder and the zero position were adjusted, and enzyme was added. The change in fluorescence with time (a decrease at $0.5\mu\text{M}$ -ethidium bromide and enhancements at both $10\mu\text{M}$ and $25\mu\text{M}$) was recorded until it reached a constant value: to ensure that this was the reaction end-point, further *EcoRI* enzyme was added to 6.0 nM , but this caused no alteration to the fluorescence. An apparent first-order rate constant (k_f) was evaluated from the chart record.

[Ethidium bromide] (μM)	Gel assays		Fluorescence assays $k_f(\text{s}^{-1})$
	$k_a(\text{s}^{-1})$	$k_b(\text{s}^{-1})$	
0	0.0068	0.0042	—
0.5	0.0068	0.0040	0.0065
10	0.0070	0.0028	0.0063
25	0.0065	0.0018	0.0070

by increasing concentration of ethidium bromide. At $0.5\mu\text{M}$ -dye, the decrease in this apparent rate constant was negligible. Though the accuracy of kinetic analyses by gel assays is limited (see Halford *et al.*, 1980), extensive repetition of experiments under the same conditions as in Table 2 showed that the slight decrease in k_b caused by $10\mu\text{M}$ -ethidium bromide was reproducible. A larger decrease was observed at $25\mu\text{M}$ -dye.

One hypothesis for why ethidium bromide should selectively inhibit the rate at which the *EcoRI* restriction endonuclease cleaves the open-circle DNA, while not affecting the rate at which this enzyme cleaves covalently closed DNA, arises from the stoichiometry of ethidium bromide binding to DNA. At saturation with ethidium bromide, covalently closed and open-circle DNA bind one molecule of dye per 5 and 2.5 base-pairs respectively (Bauer & Vinograd, 1968); at $10\mu\text{M}$ ethidium bromide under our experimental conditions, these stoichiometries will be altered to about 6 and 3 base pairs/dye molecule. Thus on both forms of the DNA, at least one molecule of ethidium bromide should intercalate within the hexanucleotide sequence of each *EcoRI* recognition site. The DNA structure at the base-pairs adjacent to and one away from intercalated ethidium bromide is distorted (Hogan *et al.*, 1979), so it is improbable that the *EcoRI* restriction enzyme interacts with its recognition site without the prior dissociation of the dye. Our fluorescence titrations indicate that ethidium bromide and *EcoRI* enzyme cannot both bind to the same site on the DNA. But with covalently closed DNA containing about one dye molecule per six base-pairs, the dissociation of ethidium bromide from within the *EcoRI* recognition site could be followed by the re-association of that dye at any of the vacant sites on the DNA; ethidium bromide would then have a negligible effect on the free energy of *EcoRI* binding to DNA. However, with open-circle DNA containing about one dye molecule per three base-pairs, the dissociation of ethidium bromide from the *EcoRI* recognition site could not be followed by its re-association at other sites, since most of these are already filled; in this case, the binding of the *EcoRI* enzyme to its recognition site would be inhibited by the dye. The *EcoRI* restriction endonuclease displays a much greater affinity for its recognition site than for alternative sequences on DNA (Halford & Johnson, 1980), whereas ethidium bromide binds with similar affinities to all DNA sequences, though showing a slight preference for intercalation between 5'-C-G-3' (Reinhardt & Krogh, 1978), a sequence that is not present within the *EcoRI* site.

The effect of ethidium bromide on the activity of the *EcoRI* restriction enzyme cannot be due to alterations in the superhelicity of the DNA. In Table

2, the same values of k_a are reported for reactions at zero and $0.5\mu\text{M}$ -ethidium bromide (where the covalently closed DNA is negatively supercoiled; see Fig. 1) and at $10\mu\text{M}$ - and $25\mu\text{M}$ -ethidium bromide (where the covalently closed DNA is positively supercoiled). In addition, the *EcoRI* restriction enzyme has similar activities towards its recognition site on plasmid pMB9 with either negatively supercoiled DNA or linear DNA obtained by cleaving plasmid pMB9 with the *SalGI* restriction endonuclease (Halford *et al.*, 1979). Thus the interaction between the *EcoRI* restriction enzyme and its recognition site is not affected by the tertiary structure of the DNA. However, the free energy liberated by the hydrolysis of one phosphodiester bond on duplex DNA is dependent on the superhelicity of the DNA, and hence at least one rate constant for a step within the mechanism for the hydrolysis of supercoiled DNA must differ from that in the hydrolysis of relaxed DNA. But it is possible that the untwisting of supercoiled DNA substrates occurs only after the cleaved DNA has dissociated from the enzyme.

Further experiments similar to the gel assays in Table 2 were carried out under different solution conditions. Variations in the MgCl_2 concentration between 3 and 30 mM, with all other conditions held as in Table 2, caused no alterations to the relative reaction rates between the absence or presence of ethidium bromide, nor did changes to the pH from 6.5 to 7.5. However, at 150 mM-NaCl instead of 50 mM, the value of k_a was decreased by a factor of 2.5 and that of k_b by a factor of 5 in the presence of $10\mu\text{M}$ -ethidium bromide when compared with an identical reaction but lacking ethidium bromide. The equilibrium dissociation constant of the *EcoRI* restriction enzyme from its recognition site on DNA is higher at 150 mM-NaCl than at 50 mM (Halford & Johnson, 1980), whereas we have found by fluorescence titrations that ethidium bromide has the same affinity for DNA at these two NaCl concentrations. Hence the enzyme will be less effective at higher salt concentrations in driving the dissociation of the dye from the DNA.

Fluorescence assays

It has been shown that, under appropriate conditions, the *EcoRI* restriction endonuclease cleaves the covalently closed form of plasmid pMB9 to yield open-circle DNA at the same rate in the presence or absence of ethidium bromide (Table 2), that the cleavage of covalently closed DNA alters the extent of ethidium bromide binding which results in a fluorescence change (Fig. 1), and that the binding of ethidium bromide is rapid compared with the turnover of the *EcoRI* restriction enzyme (Table 1). Hence the reaction of this enzyme can be monitored by fluorescence (Fig. 3). The two records

Fig. 3. Fluorescence assays

The change in fluorescence emission with time was recorded from solutions at 22°C containing 0.72 nM-*EcoRI* restriction endonuclease, 0.56 nM-DNA from plasmid pMB9 (initially 81% covalently closed), 50 mM-Tes, 50 mM-NaCl, 10 mM-MgCl₂, 0.01% gelatin, pH 7.0, and the following concentrations of ethidium bromide: (a) 0.5 μM; (b) 10 μM. The reactions were initiated by the addition of enzyme at the time noted by zero on the abscissa. The fluorescence scale is in the arbitrary units of the fluorimeter, with the same sensitivity for both reactions.

shown in Fig. 3 are from reactions differing only in the concentration of ethidium bromide: both reactions follow exponential progress curves with the same apparent rate constant (0.019 s⁻¹), but the amplitudes differ in sign in the manner expected from Fig. 1.

By theory, the fluorescence from these reactions should change with the hydrolysis of the first phosphodiester bond of the DNA, the reaction in which the *EcoRI* restriction enzyme cleaves the

covalently closed DNA to yield open circles (eqn. 1). Both open-circle and linear DNA should bind ethidium bromide to the same extent and yield the same fluorescence. To test this point, the fluorescence was monitored after the addition of *EcoRI* enzyme to the covalently closed form of plasmid pMB9 with ethidium bromide, in reaction mixtures identical with those used previously for gel assays (Table 2). For each reaction, the change of fluorescence with time (either increasing or decreasing, depending on the ethidium bromide concentration) to the reaction end-point could be described with one apparent first-order rate-constant (k_f). Values for k_f at different dye concentrations are given in Table 2: they agree well with the apparent rate constants (k_a) from gel assays for the reaction in which the *EcoRI* restriction endonuclease cleaves the covalently closed DNA to yield open circles. Thus the fluorescence signal occurs with the first reaction in eqn. (1). The fact that high concentrations of ethidium bromide can inhibit the second reaction in eqn. (1), the conversion of open circles into linear DNA (k_b in Table 2), is immaterial to the fluorescence assay, since the second reaction causes no change in fluorescence.

The amplitudes of the fluorescence change between the covalently closed and open-circle forms of the DNA that we have used to monitor the reaction of the *EcoRI* restriction endonuclease are small compared with the total fluorescence from these samples (see Fig. 2). But these are still large enough to yield reaction records comparatively free from noise, provided that a fluorimeter with suitable backing-off and scale-expansion facilities is used. The noise on the record shown in Fig. 3(b) is equal to the amplitude of the fluorescence signal that would be obtained on the hydrolysis of 13 pM-covalently closed DNA from plasmid pMB9, and that in Fig. 3(a) corresponds to that with 25 pM-DNA. For routine assays of the *EcoRI* enzyme, we have found 10 μM-ethidium bromide to be the most suitable dye concentration: at low concentrations of dye, a change in the reaction conditions can alter the sign of the fluorescence signal, whereas concentrations of ethidium bromide above 10 μM produce large fluorescence backgrounds without proportionally increasing the fluorescence change for the reaction itself (Fig. 1). Considerably larger differences in fluorescence caused by ethidium bromide binding to either covalently closed or open-circle DNA were obtained by Morgan *et al.* (1979), but their procedure cannot be adapted to yield a continuous record of an enzymic reaction.

We have used the continuous fluorescence assay to monitor the reaction of the *EcoRI* restriction endonuclease on the covalently closed form of plasmid pMB9, either under conditions with both enzyme and DNA concentrations below the K_m

value or under steady-state conditions. Under the former conditions, the complete record of the reaction was obtained up to its end-point (see Fig. 3), from which an apparent first-order rate constant (k_f) was calculated. For reactions with both enzyme and DNA concentrations below K_m but regardless of which reactant concentration was higher, the values of k_f were found to be linearly related to the concentration of enzyme, $[E_0]$: from Halford *et al.* (1980), k_f should be equal to $(k_{cat.}/K_m) \times [E_0]$. This procedure yielded values for $k_{cat.}/K_m$ of $2.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ from solutions with the same buffer, salt and MgCl_2 as in Fig. 3, and $3.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ under these conditions except for the MgCl_2 concentration being altered to 15 mM. In solutions of the latter composition, $k_{cat.}/K_m$ for the reaction of the *EcoRI* restriction enzyme against individual recognition sites on the DNA from bacteriophage λ had previously been evaluated as $1.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Halford *et al.*, 1980). But in the previous work, the assay employed on phage- λ DNA yielded a $k_{cat.}/K_m$ value for the second of the two reactions in eqn.(1) whereas the fluorescence assay used here determines $k_{cat.}/K_m$ for the first reaction: the 2-fold difference is thus the expected behaviour.

In steady-state fluorescence assays, with the *EcoRI* enzyme at a lower concentration than the DNA, the initial zero-order rates of product formation were found to decline rapidly from linearity even when the initial substrate concentration was higher than the K_m value. The decline in the steady-state rates is probably due to the open-circle form of the DNA accumulating during the reaction, since it is the first product, but this DNA remains a substrate of the *EcoRI* enzyme for the cleavage of the second strand of the duplex. Since this second reaction produces no fluorescence signal, the open-circle DNA is effectively a competitive inhibitor of the fluorescence assay. In order to use the fluorescence assay for steady-state kinetics, it was necessary to employ a high scale expansion on the recording fluorimeter so that zero-order rates were obtained from the first 20% of the reaction. We have measured the initial rates of the reaction of the *EcoRI* restriction endonuclease on the complex between the covalently closed DNA from plasmid pMB9 and ethidium bromide, over a range of DNA concentrations and with a series of different MgCl_2 concentrations. Typical data are shown in Fig. 4. At a constant enzyme concentration low enough to achieve a steady state, and at each individual concentration of MgCl_2 tested, the reaction velocities varied as a hyperbola with the DNA concentrations. Values of $k_{cat.}$ (at an infinite DNA concentration) and K_m (referring to the DNA) were calculated from these data at each MgCl_2 concentration (Fig. 5). The separate values of $k_{cat.}$ and K_m yielded values for $k_{cat.}/K_m$ of 2.7×10^7 and

Fig. 4. Steady-state kinetics

Reaction mixtures at 22°C contained the concentrations of covalently closed DNA from plasmid pMB9 shown, 0.12 nM-*EcoRI* restriction endonuclease (added last), 50 mM-Tes, 50 mM-NaCl, 10 μM -ethidium bromide, 0.01% gelatin, pH 7.0, and either (●) 10 mM or (○) 20 mM- MgCl_2 . The initial linear rate of change of fluorescence was measured from the first 20% (or less) of each reaction. Further *EcoRI* restriction endonuclease was added (final concn. 6.0 nM) to each reaction mixture, the reaction allowed to proceed to completion and the fluorescence difference between the start and the end-point of the reaction recorded. Steady-state velocities (v , nm/min) were calculated from the initial rates of fluorescence change by using the fluorescence signal corresponding to the complete reaction at each DNA concentration. The lines drawn are theoretical lines for Michaelis-Menten kinetics: at 10 mM- MgCl_2 (●), $V_{max.} = 0.48 \text{ nm/min}$ and $K_m = 2.5 \text{ nM}$; at 20 mM- MgCl_2 (○), $V_{max.} = 0.92 \text{ nm/min}$ and $K_m = 3.4 \text{ nM}$.

$3.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 10 mM- and 15 mM- MgCl_2 respectively, in good agreement with the $k_{cat.}/K_m$ values determined from the exponential reactions described above.

The data in Fig. 5 distinguish between certain mechanisms for the binding of DNA and of Mg^{2+} to the *EcoRI* restriction enzyme, the three simplest being:

where M, E and S are Mg^{2+} , the *EcoRI* enzyme and its recognition site on DNA: the product, P, is the DNA cleaved in a single strand. We assume that the cleavage of DNA occurs in a ternary complex containing both DNA and Mg^{2+} bound to the enzyme, and eqns. (2)–(4) differ only in the nature of

Fig. 5. Mg^{2+} dependency

Steady-state velocities (v) were measured as described in Fig. 4 with a series of reaction mixtures at 22°C, each covering a range of concentrations of covalently closed DNA from plasmid pMB9 ($[S]$, 0.5–8.0 nM) with 0.12 nM-*EcoRI* restriction endonuclease, 50 mM-Tes, 50 mM-NaCl, 10 μ M-ethidium bromide, 0.01% gelatin, pH 7.0, and one of the $MgCl_2$ concentrations shown. For each series, values of k_{cat} and K_m at a particular $MgCl_2$ concentration were calculated by linear regression on plots of $[S]/v$ against $[S]$; these plots are not shown, but all had correlation coefficients >0.96 . Upper plot shown is of k_{cat} values (O) and lower plot K_m values (●). The point (■) on the lower plot at zero $MgCl_2$ is the value of the equilibrium dissociation constant of the *EcoRI* restriction endonuclease from the covalently closed DNA of plasmid pMB9 measured as in Halford & Johnson (1980) with the same solution conditions as above but in the absence of both $MgCl_2$ and gelatin and the presence of 1.0 mM-EDTA.

the preceding binary complex. For each mechanism, equations were derived to relate k_{cat} and K_m to $[M]$, the concentration of Mg^{2+} . For example, it follows from eqn. (2) that:

$$k_{cat} = \frac{k_{+3}}{1 + \frac{K_c}{[M]}} \quad (5)$$

and

$$K_m = \frac{k_{+3} + \frac{k_{-1} \cdot K_c}{[M]}}{k_{+1} + \frac{k_{+1} \cdot K_c}{[M]}} \quad (6)$$

where $K_c = (k_{-2} + k_{+3})/k_{+2}$, a parameter related to

the binding of the Mg^{2+} cation to the enzyme–DNA complex. Hence if the data are consistent with eqn. (2), the dependence of k_{cat} on $[M]$ should follow a hyperbola. The values of k_{cat} were found to increase linearly with increasing $MgCl_2$ up to 20 mM (Fig. 5); this is consistent with eqn. (5) only if $K_c > 20$ mM. [The values of k_{cat} decline above 20 mM- $MgCl_2$. However, the activity of the *EcoRI* restriction enzyme is decreased by high ionic strength (Halford *et al.*, 1980), so this may be a non-specific effect and has not been analysed further]. From eqn. (6), the values of K_m for DNA in eqn. (2) should change with the Mg^{2+} concentration from k_{-1}/k_{+1} at $[M] = 0$ to k_{+3}/k_{+1} when $[M] \gg K_c$. Thus the observed increase of K_m with increasing $MgCl_2$ (Fig. 5) demands that $k_{-1} < k_{+3}$; this requirement concurs with the suggestion of Halford *et al.* (1980) that, under these experimental conditions (50 mM-salt, pH 7.0), the rate constant for the dissociation of the enzyme from the DNA–enzyme binary complex is smaller than that for the hydrolysis of enzyme-bound DNA. Moreover, the equilibrium dissociation constant of the enzyme from its recognition site on DNA (k_{-1}/k_{+1} in eqn. 2) has been evaluated as 0.5 nM under these same conditions but without $MgCl_2$ (Halford & Johnson, 1980): the values of K_m determined at low $MgCl_2$ concentrations approach the value of this equilibrium dissociation constant (Fig. 5), as is required by eqn. (6).

Thus the available data (except that at 30 mM- $MgCl_2$) are consistent with the reaction mechanism given in eqn. (2), in which the enzyme binds first to the DNA and then Mg^{2+} binds with a low affinity to the enzyme–DNA complex. The mechanism in eqn. (3) can be discounted, since it predicts that k_{cat} should be invariant with the Mg^{2+} concentration when $[M] \gg [S]$ (Dixon & Webb, 1964): this prediction is contrary to the experimental data in Fig. 5. But the mechanism in eqn. (4) cannot be discounted so rigorously. However, in order to accommodate the data in Fig. 5 to the equations for k_{cat} and K_m from the mechanism in eqn. (4), it is necessary to postulate that its rate-limiting step is the binding of Mg^{2+} to the free enzyme: if any other step in eqn. (4) is rate-limiting, k_{cat} will be independent of $[M]$. This is improbable because the concentrations of $MgCl_2$ in our experiments are much greater than those of the other reactants. In addition, though equilibrium binding studies have indicated that either Mg^{2+} or DNA can bind to the free enzyme, the binary complex between Mg^{2+} and the *EcoRI* enzyme cannot readily bind DNA and is perhaps an abortive complex (Halford & Johnson, 1980). Hence the mechanism in eqn. (2) is preferred to the alternatives given in eqns. (3) and (4), but it is certain that other reaction mechanisms, containing more intermediates, could be found that are also consistent with the data.

The steady-state kinetics presented here cannot readily be compared with previous measurements of k_{cat} and K_m for the *EcoRI* restriction endonuclease (Greene *et al.*, 1975; Modrich & Zabel, 1976; Woodhead & Malcolm, 1980; Langowski *et al.*, 1980); each study employed different DNA molecules as the substrate, different reaction conditions (with only one Mg^{2+} concentration in each previous study) and different assay procedures (where some measure the rate of cleavage of the first of the two strands in the DNA and others the second). Halford *et al.* (1979) had previously estimated by an indirect procedure a value of $>13.5 \text{ nm}$ for the K_m of the *EcoRI* restriction enzyme with plasmid pMB9 in $50 \text{ mM-Tris}/100 \text{ mM-NaCl}/5 \text{ mM-MgCl}_2$, pH 7.5. With these conditions, reaction velocities were found to vary linearly with increasing DNA concentrations up to 6.0 nm , so here the K_m value must be greater than that measured at 5 mM-MgCl_2 under the conditions used in Fig. 5. Values of K_m for the *EcoRI* restriction enzyme are generally quoted as concentrations of duplex DNA. But if the substrate concentrations in Fig. 4 had been given in terms of strands of DNA, since an intact *EcoRI* recognition site has the same 5'-3' nucleotide sequence in both strands, the K_m values in Fig. 5 would have been doubled.

Applications

The fluorescence assay outlined has several advantages in studies of the *EcoRI* restriction endonuclease when compared with assays that involve the separation of the reaction products from the substrate by electrophoresis. Firstly it is less laborious to measure a reaction rate on a recording spectrofluorimeter than to take numerous samples from the reaction at timed intervals, subject each to gel electrophoresis and then determine the concentrations of substrate and product by scintillation counting or other methods. Secondly, the fluorescence method can have a higher sensitivity than the gel methods. The minimum concentrations of DNA from plasmid pMB9 that are needed to yield reaction profiles with signal-to-noise ratios of 10:1 are 0.15 nm in fluorescence assays (Fig. 3b) and 1.5 nm in gel assays using our preparations of $[^3\text{H}]\text{DNA}$. (However, the specific radioactivity of the DNA preparations could be increased by using more $[^3\text{H}]\text{thymidine}$. Alternatively, a higher sensitivity in terms of DNA molarities could be obtained for both fluorescence and gel assays by using a DNA molecule of higher molecular weight than plasmid pMB9).

But the principal advantage of our fluorescence assay is that it yields a continuous record of the reaction, where all previous methods for restriction-enzyme assays give only the amount of product formation at a single time point. In some studies on

the *EcoRI* restriction enzyme, reaction velocities have been calculated from the amount of product formed within a solitary time point on each reaction (Modrich & Zabel, 1976; Langowski *et al.*, 1980). More accurate evaluations of the reaction rates would have been obtained with continuous records. Moreover, since the covalently closed DNA from plasmid pMB9 is not sheared by passage through the stopped-flow fluorimeter and the binding of ethidium bromide to DNA is rapid (Table 1), the fluorescence assay could also be used in transient kinetic experiments to study processes with half times as short as 50 ms. It is immaterial for steady-state kinetics, but not for transient kinetics, which individual step within the reaction mechanism of the *EcoRI* enzyme produces the fluorescence signal.

Two disadvantages of the fluorescence assay must be noted. Firstly, the assay demands preparations of covalently closed DNA substrates containing low amounts of either open-circle or linear DNA, since the latter decrease the rate of fluorescence change. Secondly, the cleavage of any phosphodiester bond on the covalently closed DNA will yield the same fluorescence change with ethidium bromide. Hence a preparation of a restriction endonuclease contaminated by a non-specific endonuclease could not be assayed by this procedure, but this also applies to several other assays. Likewise, a covalently closed DNA molecule containing two or more copies of the recognition site for the restriction enzyme could not be used as a substrate, since it would be impossible to determine from fluorescence which site had been cleaved. Plasmids in multiple copies within the bacterial cell can undergo general recombination to produce DNA molecules containing two or more copies of the complete DNA sequence of the plasmid, but these multimeric forms are not produced in *recA* strains of *E. coli* (Potter & Dressler, 1976).

The continuous fluorescence assay developed here for studies on the *EcoRI* restriction endonuclease could also be applied to many enzymes that alter the structure of covalently closed DNA such as other endonucleases or topoisomerases. For each enzyme, it would be necessary to determine whether or not that enzyme displayed the same activity towards the DNA in the presence or absence of ethidium bromide. If a high concentration of ethidium bromide, which will introduce positive supercoils into the covalently closed DNA, is unsuitable for a particular enzyme, the assay will still operate at low concentrations of the dye that leaves the DNA with negative supercoils (Fig. 3a). Champoux & McConaughy (1975) monitored by ethidium bromide fluorescence the decrease in the positive superhelicity of covalently closed DNA caused by the rat liver DNA topoisomerase, but did not show whether the rates observed by fluorescence corresponded to

the enzymic rates in the absence of ethidium bromide. The only enzyme apart from *EcoRI* that we have tested is the *SalGI* restriction endonuclease. The recognition sequence for this enzyme occurs once on plasmid pMB9. Under the conditions 50 mM-Tris/125 mM-NaCl/10 mM- β -mercaptoethanol, 500 μ g of heat-denatured bovine serum albumin/ml, with either 10 mM- or 0.5 mM-MgCl₂, pH 8.0 at 22°C, the *SalGI* restriction endonuclease cleaved the covalently closed DNA of plasmid pMB9 at the same rate in the presence of ethidium concentrations up to 10 μ M as in its absence. Under these conditions with 10 mM-MgCl₂, the *SalGI* enzyme cleaves both strands of this DNA in a concerted reaction to yield the linear DNA, but at 0.5 mM-MgCl₂, either one or both strands are cleaved in two parallel reactions that produce either open-circle or linear DNA (Maxwell & Halford, 1981). None of these reactions are affected by the concentrations of ethidium bromide tested.

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