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Modes of DNA Cleavage by the *Eco*RV Restriction Endonuclease†

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ABSTRACT: The mechanism of action of the *Eco*RV restriction endonuclease at its single recognition site on the plasmid pAT153 was analyzed by kinetic methods. In reactions at pH 7.5, close to the optimum for this enzyme, both strands of the DNA were cut in a single concerted reaction: DNA cut in only one strand of the duplex was neither liberated from the enzyme during the catalytic turnover nor accumulated as a steady-state intermediate. In contrast, reactions at pH 6.0 involved the sequential cutting of the two strands of the DNA. Under these conditions, DNA cut in a single strand was an obligatory intermediate in the reaction pathway and a fraction of the nicked DNA dissociated from the enzyme during the turnover. The different reaction profiles are shown to be consistent with a single mechanism in which the kinetic activity of each subunit of the dimeric protein is governed by its affinity for Mg^{2+} ions. At pH 7.5, Mg^{2+} is bound to both subunits of the dimer for virtually the complete period of the catalytic turnover, while at pH 6.0 Mg^{2+} is bound transiently to one subunit at a time. The kinetics of the *Eco*RV nuclease were unaffected by DNA supercoiling.

Class II restriction endonucleases recognize specific base sequences in duplex DNA, typically 4–6 bp[1] long, and cleave both strands of the DNA at fixed locations relative to their recognition sites. The only cofactor that they need for phosphodiester hydrolysis is Mg^{2+} ions. These enzymes thus provide test systems for analyzing how a protein can catalyze a reaction at a specific site on DNA. However, different restriction enzymes can display very different reaction mechanisms (Halford et al., 1979; Potter & Eckstein, 1984). At present, at least 615 restriction enzymes have been identified (Kessler & Holtke, 1986) but only 3, *Eco*RI, *Eco*RV, and *Hha*II, have been crystallized to date (Rosenberg et al., 1978; D'Arcy et al., 1985; Chandrasegeran et al., 1986). For the *Eco*RI endonuclease, the structure of its recognition complex with DNA has been elucidated at high resolution (McClarin et al., 1986), and its mechanism of action in solution has been studied extensively (Modrich, 1982; Halford, 1983). However, high-resolution structures of both *Eco*RV and *Hha*II have yet to be reported, though the mechanism of action of *Hha*II has already been analyzed (Kaddurah-Daouk et al., 1985). We describe here studies on the mechanism of action of the *Eco*RV enzyme.

The *Eco*RV restriction endonuclease cleaves DNA specifically at the sequence

5′-G-A-T↓A-T-C-3′
3′-C-T-A↑T-A-G-5′

at the sites marked by arrows (Schildkraut et al., 1984; D'Arcy et al., 1985). Under standard reaction conditions, all other DNA sequences, even those differing by only one bp, are cut at least 10^4 times more slowly (Halford et al., 1986). The amino acid sequence of the *Eco*RV nuclease predicts a polypeptide of M_r 28 600 (Bougueleret et al., 1984). In solution, it exists as a dimer of two such subunits (D'Arcy et al., 1985; Luke et al., 1987). The size and subunit structure of the *Eco*RV enzyme is thus similar to those of *Eco*RI and many other class II restriction enzymes (Modrich, 1982). However, *Eco*RV not only cleaves DNA at a different site from *Eco*RI

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[1] Abbreviations: bp, base pairs; M_r, relative molecular mass; BME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; K_D, equilibrium dissociation constant.

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but also has no amino acid sequence homology with *Eco*RI (Bougueleret al., 1984). Preliminary studies on the kinetic mechanism of the *Eco*RV nuclease were reported by Luke et al. (1987).

Experimental Procedures

Proteins. A strain to overproduce the *Eco*RV restriction enzyme, *Escherichia coli* 1100 [pTZ115-14, pLBM, pRK248], was provided by M. Zabeau (P.G.S., Ghent, Belgium); the strain is that described by Bougueleret et al. (1985) except that pTZ115-14 was used in place of pTZ115 to generate more of the *Eco*RV enzyme (Botterman et al., 1985). Growth of the strain with induction for *Eco*RV was as in Bougueleret et al. (1985). After disruption of the cells by sonication, the *Eco*RV enzyme was purified to >99% homogeneity by chromatography on phosphocellulose (Whatman P11) and Blue Sepharose CL-6B (Pharmacia) as described by Luke et al. (1987). Protein concentrations were measured from the recovery of amino acids (relative to an internal standard of norleucine) on a Rank Hilger J180 amino acid analyzer after 24-h hydrolyses in 6 M HCl with 0.1% phenol at 105 °C in vacuo. Molarities of the *Eco*RV restriction enzyme are given in terms of the dimeric protein of M_r 57 000.

Stock solutions of the purified restriction enzyme in 10 mM K_2HPO_4, 100 mM NaCl, 5 mM BME, 0.5 mM EDTA, and 50% (v/v) glycerol, pH 7.0 (containing typically 5–15 μM *Eco*RV), were stored at −20 °C: full activity was retained after 18 months. Prior to each reaction, a sample of the stock of *Eco*RV was diluted to yield the requisite concentration (typically 10 nM) in 50 mM Tris, 100 mM NaCl, 10 mM BME, 1 mM spermine, and 100 μg/mL BSA, pH 7.5; diluted enzyme in this buffer retained full activity over 1 h at 20 °C.

Topoisomerase I (from calf thymus) and restriction endonuclease *Pst*I were obtained from Bethesda Research Laboratories (Paisley, U.K.) and used as advised by the supplier. Conversion of supercoiled DNA to the relaxed covalently closed form by topoisomerase I was checked by electrophoresis of the DNA through agarose in the presence of chloroquine (Shure et al., 1977). BSA and spermine were obtained from Sigma (London, U.K.); solutions of BSA were heated at 67 °C for 16 h to inactivate contaminating nucleases.

DNA. E. coli HB101 (Boyer & Roulland-Dussoix, 1969), transformed with the monomeric form of the plasmid pAT153 (Twigg & Sherratt, 1980), were grown to late log phase in M9 minimal media with 0.2% (w/v) casamino acids at 37 °C before addition of chloramphenicol to 150 μg/mL, 2-deoxyadenosine to 250 μg/mL, and [*methyl*-^{3}H]thymidine to 1.25 μCi/mL. After continued growth at 37 °C overnight, cells were harvested by centrifugation and the covalently closed form of the plasmid was purified by a method that includes density gradient centrifugations at two different concentrations of ethidium bromide (Halford & Johnson, 1981). After removal of ethidium bromide with CsCl-saturated propan-2-ol, the DNA was dialyzed against 10 mM Tris and 0.1 mM EDTA, pH 7.5, and stored at 4 °C. Concentrations of DNA were evaluated from UV absorption at 260 nm, and molarities of pAT153 are given in terms of the duplex DNA molecule of M_r 2.42 × 10^6. This is equal to the molarity of *Eco*RV recognition sites.

Kinetic Methods. Steady-state kinetics were typically carried out by diluting the stock solution of *Eco*RV nuclease to 10 nM enzyme as above and then adding a sample of the diluted enzyme (10 μL) to a solution of ^{3}H-labeled pAT153 (190 μL) in buffer at 20 °C. Compositions of solutions given under Results and Discussion omit components from the enzyme dilution buffer. Samples (10 μL) were removed from the reaction mixture at timed intervals and immediately vortexed with 5 μL of stop-mix (0.1 M EDTA, 0.1 M Tris, 40% (w/v) sucrose, and 100 μg/mL bromophenol blue, pH 8.0). Each sample was then analyzed by electrophoresis through 1.2% agarose in Tris–acetate containing 0.5 μg/mL ethidium bromide at 8 V/cm: under these conditions (Johnson & Grossman, 1977), the covalently closed form of pAT153 has a higher mobility than the linear form, which in turn has a higher mobility than the open-circle form of this DNA. For each sample from the reaction, slices of the gel that covered each of the three forms of the DNA were excised, dissolved by incubation for 1 h at 67 °C in 0.5 mL of 5 M $NaClO_4$, and added to 3 mL of water and 10 mL of ES299 scintillant (Canberra-Packard, Pangbourne, U.K.), and the radioactivity in each was determined by scintillation counting. Values for the amount of each form of the DNA were calculated as a percentage of the total DNA in that sample.

Transient and single turnover kinetics were carried out by holding a sample of one reagent (10 μL) on a vortex mixer and then adding the second reagent (10 μL) followed by 10 μL of stop-mix; the time interval between addition of the second reagent and the stop-mix was recorded by stopwatch. Both reagents were at 20 °C prior to the reaction. Samples were subsequently analyzed as above. For single turnovers at pH 6.0, the enzyme dilution buffer noted above was modified by including 50 mM Mes, pH 6.0, in place of Tris, pH 7.5.

For both steady-state and transient kinetic experiments, zero time points were taken by adding stop-mix to a sample of the DNA before the enzyme. The preparations of pAT153 were usually about 90% covalently closed DNA and 10% open-circle form, the latter being created by radiolysis or other nonspecific nicking during the storage of each preparation. Values for the formation of open-circle DNA during reactions with the *Eco*RV nuclease have been corrected by subtracting the level at zero time. For every reaction, a sample from the same solution of DNA was also incubated with enzyme dilution buffer (but lacking *Eco*RV) for the duration of the reaction. In all of the reactions described here, <2% of the covalently closed DNA was nicked in this control; data from reactions where this control yielded >2% nicking were discarded as one or more of the solutions must then have been contaminated with a nonspecific nuclease. For some experiments, samples from the reactions were mixed with phenol instead of the above stop-mix: these yielded the same level of product formation per time as the EDTA stop-mix, and hence both reagents must terminate the activity of the *Eco*RV nuclease very rapidly relative to the time scales being considered here.

Results and Discussion

Experimental Conditions. As a substrate for the *Eco*RV restriction endonuclease, we used the covalently closed form of the plasmid pAT153 (Twigg & Sherratt, 1980). This is a circular DNA molecule of 3658 bp of known sequence that differs from pBR322 (Sutliffe, 1978) by one deletion of 705 bp. The monomeric form of pAT153 was maintained in a *rec*A strain of *E. coli* in order to prevent recombination from yielding multimers with tandem repeats of its DNA sequence. The monomer has one *Eco*RV recognition sequence, and under the reaction conditions used here, virtually all of the observed cutting of this DNA by the *Eco*RV enzyme will be at this site; cleavage of other sequences is much slower (Halford et al., 1986). The reaction of the *Eco*RV nuclease on the covalently closed form of pAT153 could yield initially either the open-circle form by cutting one strand of the DNA at the recognition site or linear DNA by cutting both strands: in the

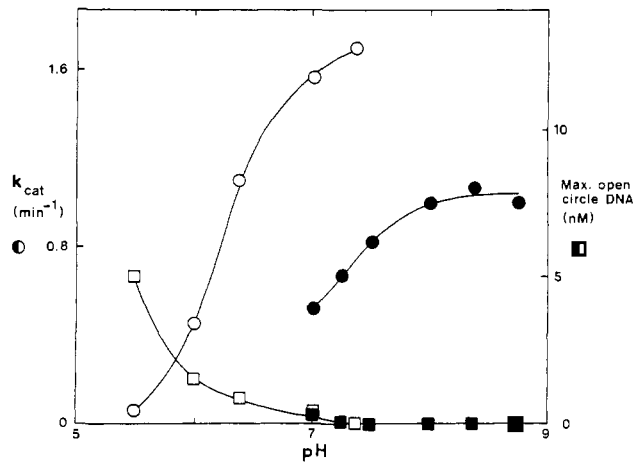

FIGURE 1: pH profile. Reactions at 20 °C contained 0.5 nM *Eco*RV restriction endonuclease, 10 nM pAT153 (^{3}H-labeled) with 10 mM $MgCl_2$ in 50 mM buffer (specified below), 100 mM NaCl, and 10 mM BME at the pH indicated. Buffer was either Tris (●, ■) or Mes (○, □). At >5 time points in each reaction, the amounts of the covalently closed, open-circle, and linear forms of pAT153 were determined as under Experimental Procedures. Values for k_{cat} (●, ○; left-hand ordinate) were measured from the initial rate at which the concentration of the covalently closed DNA declined. The maximal amount of open-circle DNA formed during each reaction (■, □; right-hand ordinate) was also measured.

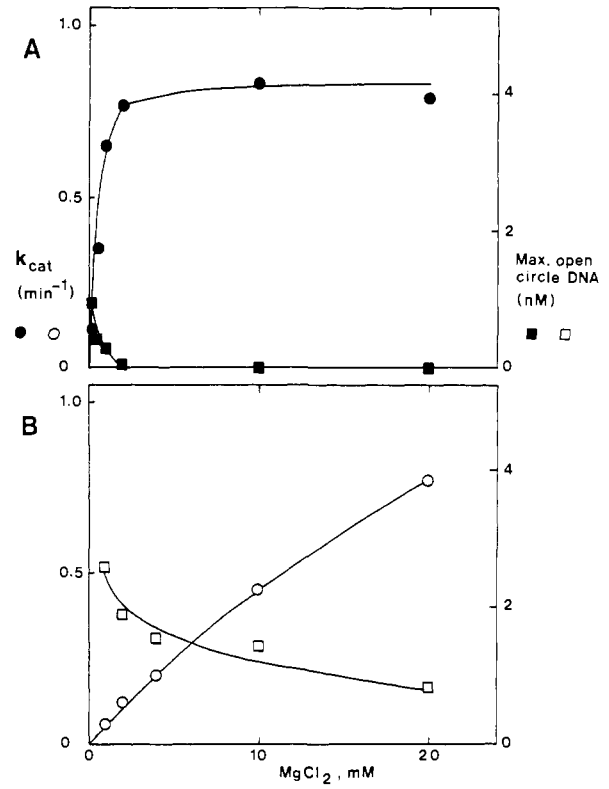

FIGURE 2: Mg^{2+} dependence. Reactions at 20 °C contained 0.5 nM *Eco*RV restriction endonuclease, 10 nM pAT153 (^{3}H-labeled), 100 mM NaCl, and 10 mM BME, in either 50 mM Tris, pH 7.5 (●, ■; plot A), or 50 mM Mes, pH 6.0 (○, □; plot B), and the concentration of $MgCl_2$ indicated. Evaluations of k_{cat} (●, ○; left-hand ordinate) and maximal open-circle formation (■, □; right-hand ordinate) were as in Figure 1.

former case, a second reaction would subsequently convert the open circle to the linear form. The three forms of DNA can be separated from each other by electrophoresis through agarose (Johnson & Grossman, 1977). As in previous studies on other restriction enzymes (Halford, 1983), we have used this method with ^{3}H-labeled DNA to provide a quantitative analysis of the kinetics of *Eco*RV.

Variations in the activity of the *Eco*RV restriction enzyme with reaction conditions were identified by steady-state kinetics with reactions that contained pAT153 in large excess over the *Eco*RV enzyme. For each set of conditions tested, the decline in the concentration of the covalently closed form of the plasmid remained linear with time until over half of the substrate had been cleaved. The initial zero-order rates varied linearly with the concentration of *Eco*RV enzyme and were normalized against the enzyme concentrations to yield k_{cat} values. However, the plasmid pAT153 has in addition to its *Eco*RV recognition sequence a further 3652 bp of DNA. With other restriction enzymes, the DNA outside the recognition site can act as a competitive inhibitor of the reaction at the recognition site (Langowski et al., 1980; Maxwell & Halford, 1982b), and this might also occur with the *Eco*RV enzyme. Hence, all of the kinetic parameters for *Eco*RV noted here are apparent values measured at a fixed ratio of substrate to inhibitor.

Figure 1 shows the variation of k_{cat} with pH in reactions containing constant levels of NaCl (100 mM) and $MgCl_2$ (10 mM). The *Eco*RV restriction enzyme shows a large specific buffer effect, with higher rates in Mes than in Tris. Reactions at pH values above 8.5 also had a specific buffer effect, with higher rates in Ches than in Tris (data not shown). Mes and Ches are both anionic buffers, and the activity of *Eco*RV is known to vary between solutions with different anions (Lerimo et al., 1987). However, in both Mes and Tris buffers, acidic pH values reduced k_{cat}. In either Mes at pH 6.0 or Tris at pH 7.5, with the same constant level of NaCl and $MgCl_2$ as above, steady-state rates were measured as a function of the concentration of the DNA substrate. No variation in rate was observed across the range tested, 2–30 nM pAT153, at either pH (data not shown). Hence, at both pH 6.0 and pH 7.5, the K_m of the *Eco*RV restriction enzyme for pAT153 must be ≪2 nM, and the decline in activity at acidic pH values cannot be due to weakened binding of the enzyme to DNA.

At pH 7.5 and 10 mM $MgCl_2$, the activity of the *Eco*RV nuclease increased with increasing concentrations of NaCl from 0 to 100 mM and decreased at higher concentrations of salt (data not shown). Values of k_{cat} at 50 and 150 mM NaCl were, respectively, 75% and 40% of that at 100 mM NaCl. For the *Eco*RV enzyme, we have not tested whether the optimal conditions, with respect to salt and pH, vary between DNA molecules as happens with *Eco*RI (Halford & Johnson, 1980).

Either Mg^{2+} or certain other divalent metal ions (Luke et al., 1987) are essential for the activity of the *Eco*RV restriction enzyme. However, the variation in k_{cat} with the concentration of $MgCl_2$ observed in reactions with Tris at pH 7.5 differs markedly from that in Mes at pH 6.0 (Figure 2). At pH 7.5, k_{cat} was essentially invariant with concentrations of $MgCl_2$ above 2 mM and the level of $MgCl_2$ needed to achieve half of this maximal rate was about 0.5 mM. The latter concentration of $MgCl_2$ is of the same order of magnitude as the concentration of phosphate groups from 10 nM pAT153, and thus, without knowing the concentration of free Mg^{2+}, we cannot evaluate an apparent K_D for Mg^{2+} but it must be <0.5 mM. In contrast, the Mg^{2+} dependence of k_{cat} at pH 6.0 yielded values of about 25 mM for the apparent K_D for Mg^{2+} and of about 1.7 min^{-1} for k_{cat} when saturated with Mg^{2+}. These values were determined by an Eadie plot (not shown) of the data in Figure 2B, but given the lack of data from reactions with concentrations of $MgCl_2$ in large excess of this value for K_D, they must be considered as approximations. However, it is striking that the value of 1.7 min^{-1} for k_{cat} in

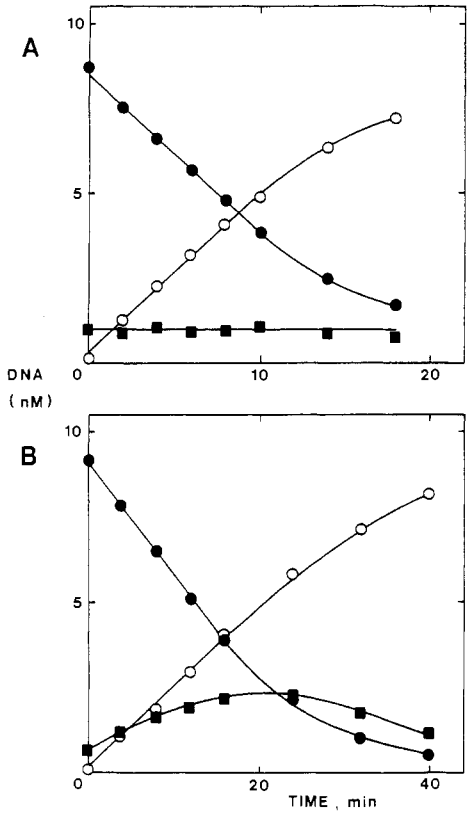

FIGURE 3: Steady-state profiles. Samples were withdrawn at the indicated times from reactions at 20 °C that contained 0.5 nM *Eco*RV restriction endonuclease, 10 nM pAT153 (^{3}H-labeled), and 10 mM $MgCl_2$ in 50 mM buffer (specified below), 100 mM NaCl, and 10 mM BME, quenched immediately, and subsequently analyzed for the amounts of covalently closed (●), open-circle (■), and linear forms (○) of pAT153. Buffers were either Tris, pH 7.5 (plot A), or Mes, pH 6.0 (plot B).

Mes at pH 6.0 at a saturating concentration of $MgCl_2$ is similar to that observed in Mes at pH values above 7.0 with 10 mM $MgCl_2$ (Figure 1).

Hence, the decreased activity of the *Eco*RV restriction enzyme at acidic pH values (Figure 1) may be due solely to a reduction in the affinity of the system for Mg^{2+}, with 10 mM $MgCl_2$ (as used for the experiments in Figure 1) being sufficient to saturate the enzyme at pH values above 7 but being insufficient at pH values below 7. The rate constants for catalysis by the *Eco*RV–Mg^{2+} complex may be independent of pH over the range tested.

Steady-State Profiles. Values for k_{cat} in Figures 1 and 2 were measured from the initial rate at which the concentration of the covalently closed form of pAT153 declined during these reactions. However, the initial product that *Eco*RV generated from the covalently closed DNA varied with the reaction conditions (Figure 3). At pH 7.5 and 10 mM $MgCl_2$, *Eco*RV converted the covalently closed DNA directly to the linear form: none of the open-circle form was observed during this steady-state reaction (Figure 3A). This profile is consistent with a concerted mechanism in which the *Eco*RV nuclease cuts both strands of the DNA during the lifetime of a single enzyme–DNA complex.

In contrast, at pH 6.0 and 10 mM $MgCl_2$, the *Eco*RV restriction enzyme must have initially cleaved at least some of the DNA in only one strand of the recognition site, for open circles of pAT153 were produced during the reaction (Figure 3B). In the reaction shown, the open-circle DNA reached a maximum concentration of about 1.5 nM, which is larger than that of the *Eco*RV enzyme (0.5 nM). Hence, these open circles cannot be due solely to enzyme-bound intermediates and instead must arise from DNA that is liberated from the enzyme after a single strand is cut. However, it is probable that only a fraction of the complexes between the enzyme and DNA cut in one strand dissociate before the second strand is cut; if all of the complexes dissociated, we would expect a larger amount of open-circle DNA than that observed.

In each of the steady-state reactions of *Eco*RV in Figures 1 and 2, we measured the amount of open-circle DNA at its maximum during the reaction. The maximal levels of open-circle formation increased with either decreasing pH or decreasing concentrations of $MgCl_2$ (Figures 1 and 2). At pH 7.5, where no open-circle DNA was produced in the presence of 10 mM $MgCl_2$ (Figure (3A), a small amount of the open-circle form was seen in reactions at 0.1 mM $MgCl_2$ (Luke et al., 1987). However, open-circle DNA was not formed under all conditions that yielded suboptimal k_{cat} values. For example, in steady-state reactions at pH 7.5 and 10 mM $MgCl_2$, with either zero or 200 mM NaCl, the *Eco*RV enzyme still converted covalently closed pAT153 directly to its linear form, albeit slowly (data not shown).

Reaction profiles for class II restriction endonucleases differ considerably among these enzymes, from the concerted cleavage of both strands of the DNA through to separate and independent reactions at each strand (Potter & Eckstein, 1984). However, the steady-state profiles of the *Eco*RV enzyme shown here have marked similarities to those observed previously with the *Sal*I restriction enzyme (Maxwell & Halford, 1982a). Under its optimal reaction conditions, *Sal*I also cleaved its recognition site in both strands of the DNA by one concerted reaction. Moreover, the only reaction conditions that caused *Eco*RV to generate DNA cleaved in a single strand, either low pH or low concentrations of $MgCl_2$, were also the only conditions where *Sal*I nicked its DNA (Maxwell & Halford, 1982a).

A single mechanism may account for why two different restriction enzymes behave identically in this respect. Both *Eco*RV and *Sal*I are active as dimers of identical protein subunits (Luke et al., 1987; Maxwell & Halford, 1982a), and both recognize symmetrical DNA sequences. These enzymes will presumably form symmetrical complexes with their respective recognition sites of the type seen with *Eco*RI (McClarin et al., 1986), where half of the dimer is placed to cleave one strand of the DNA and the other half the second strand. However, each subunit of the dimer must bind Mg^{2+} before it can cleave a phosphodiester bond. Under reaction conditions where the system has a high affinity for Mg^{2+} (Figure 2A), the enzyme will have for most of the time that it is bound to DNA two ions of Mg^{2+}, one associated with each subunit. It will then be able to cut both strands of the DNA during the lifetime of a single enzyme–DNA complex (Figure 3A). In contrast, when the system has a low affinity for Mg^{2+} (Figure 2B) or when the concentration of $MgCl_2$ is very low, then for most of the time that it is bound to DNA the enzyme will be either free of Mg^{2+} or bound to just one ion of Mg^{2+} per dimer. This will increase the probability of the enzyme dissociating from the DNA before cutting it in both strands (Figure 3B). We do not know whether the affinity of *Sal*I for Mg^{2+} decreases with decreasing pH, as shown here for *Eco*RV, but it is possible that both enzymes bind Mg^{2+} at a site whose function depends on acidic amino acid side chains being unprotonated.

Single Turnover and Transient Kinetics. To test the above hypothesis, that the mode of DNA cleavage by the *Eco*RV restriction enzyme is determined primarily by its fractional

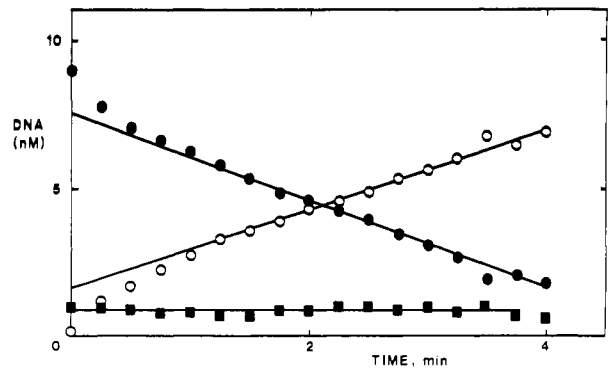

FIGURE 4: Transient kinetics at pH 7.5. Samples of enzyme and DNA at 20 °C were mixed to yield reactions that contained 2 nM *Eco*RV restriction enconuclease, 10 nM pAT153 (^{3}H-labeled), and 10 mM $MgCl_2$ in 50 mM Tris, 100 mM NaCl, and 10 mM BME, pH 7.5. The reactions were quenched at the indicated times and subsequently analyzed for the amounts of the covalently closed (●), open-circle (■), and linear forms (○) of pAT153. Each data point is the average from two separate experiments, and the lines drawn are linear regressions to all data points between 1.5 and 4.0 min.

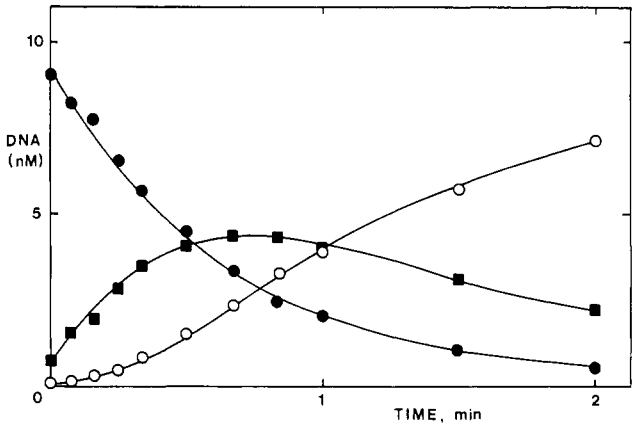

FIGURE 5: Single turnover at pH 6.0. Samples of enzyme and DNA at 20 °C were mixed to yield reactions that contained 320 nM *Eco*RV endonuclease, 10 nM pAT153 (^{3}H-labeled), and 10 mM $MgCl_2$ in 50 mM Mes, 100 mM NaCl, and 10 mM BME, pH 6.0. The reactions were quenched at the indicated times and subsequently analyzed for the amounts of the covalently closed (●), open-circle (■), and linear forms (○) of pAT153.

saturation with Mg^{2+}, we used single turnover and transient kinetic methods to analyze DNA cleavage by *Eco*RV at both pH 7.5 and pH 6.0. In order to see whether a transient phase of product formation preceded the steady state, we carried out reactions with concentrations of pAT153 higher than that of the *Eco*RV enzyme but in which the enzyme concentration was sufficiently high to allow for the detection of presteady state intermediates. Figure 4 shows data from such a reaction at pH 7.5. A transient phase was observed: during the first minute of this reaction, the amount of covalently closed form decreased more rapidly and that of the linear form increased more rapidly than in the subsequent zero-order steady-state phase. As expected [eq 4.79 in Fersht (1985)], the amplitude of the transient phase, measured after back-extrapolation of the steady-state phase to zero time, increased linearly with the concentration of *Eco*RV enzyme (data not shown). Under these experimental conditions, 10 mM $MgCl_2$ at pH 7.5, open-circle DNA was not produced during steady-state reactions at low concentrations of *Eco*RV (Figure 3A), but it is striking that the transient phase also fails to yield any of the open-circle form (Figure 4). If the concentration of open-circle DNA had reached a level equal to 25% of the enzyme concentration at any time during the reaction in Figure 4, it would readily have been detected.

DNA cleavage by the *Eco*RV restriction enzyme must involve the intermediates

$$(E_1{\cdot}E_2)(S_1{\cdot}S_2) \xrightarrow{k_1} (E_1{\cdot}E_2)(P_1{\cdot}S_2) \xrightarrow{k_2} (E_1{\cdot}E_2)(P_1{\cdot}P_2) \quad (1)$$

where (E·E) is the protein dimer, (S·S) is the DNA duplex with an intact *Eco*RV recognition site, and (P·S) and (P·P) are DNA molecules cut at the *Eco*RV site in either one or both strands. Subscripts 1 and 2 identify the individual subunits of the protein and the strands of the DNA. In the reaction at pH 7.5 (Figure 4), the absence of open-circle DNA (P·S in eq 1) during the pre-steady-state phase means that the transient is due to rapid cleavage of both strands of enzyme-bound DNA prior to the rate-limiting step of the enzyme turnover. The rate of the transient phase could be limited by k_1, the hydrolysis of the first phosphodiester bond, and if this is the case, then $k_1 \ll k_2$: without open-circle formation, it is not possible for k_2 to limit the rate of the transient phase. Alternatively, the rate of the transient phase might be limited by an earlier step in the mechanism, perhaps one associated with the initial binding of the DNA prior to the first intermediate in eq 1, and if this is the case, then the data provide no information about the relative values of k_1 and k_2. The steady-state rate for the enzyme turnover at pH 7.5 must be limited by a step that occurs after both strands of the DNA are cut, and this is most likely to be the final dissociation of the cut DNA (P·P in eq 1) from the enzyme.

A similar analysis was carried out on the reaction of the *Eco*RV enzyme at pH 6.0, illustrated in this case by a single turnover of the enzyme (Figure 5). Single turnover reactions with a 3-fold molar excess of *Eco*RV over pAT153 yielded profiles identical with those of reactions with a 30-fold excess of enzyme over DNA (data not shown). Hence, these experiments differ from steady-state reactions under these experimental conditions, when some open-circle DNA was liberated from the enzyme (Figure 3B), in that they provide information about the DNA as it is bound to the enzyme. The data in Figure 5 fit those expected for a two-step consecutive reaction with an obligatory intermediate [eq 4.31 in Fersht (1985)]. The DNA–protein complexes involved here are shown in eq 1. In the first step, the enzyme must cleave one strand of the DNA to yield the nicked intermediate, $(E_1{\cdot}E_2)(P_1{\cdot}S_2)$ in eq 1, at a rate defined by k_1. In the second step, the enzyme converts the nicked intermediate to the final product cut in both strands, at a rate defined by k_2, but only after a lag phase that confirms the consecutive nature of these reactions. Evaluation of k_1 and k_2 from single turnovers of *Eco*RV at pH 6.0 and 10 mM $MgCl_2$ yielded average values of 1.7 min^{-1} for k_1 and 0.9 min^{-1} for k_2.

The ratio of close to 2/1 for k_1/k_2 may be due simply to the statistics of Mg^{2+} binding to each subunit of a dimeric enzyme. For the mechanism

$$E{\cdot}S + Mg^{2+} \rightleftarrows E{\cdot}S{\cdot}Mg^{2+} \xrightarrow{k_c} E{\cdot}P{\cdot}Mg^{2+} \quad (2)$$

the apparent rate constant for the formation of product, k_{obsd}, is related to the microscopic constant, k_c, by

$$k_{obsd} = \frac{k_c}{1 + K_D/[Mg^{2+}]} \quad (3)$$

where K_D is the equilibrium dissociation constant for Mg^{2+}. When $K_D \gg [Mg^{2+}]$, as will be the case for *Eco*RV at pH 6.0 with 10 mM $MgCl_2$ (Figure 2B), k_{obsd} will vary linearly with the ratio $[Mg^{2+}]/K_D$. With the complex $(E_1{\cdot}E_2)(S_1{\cdot}S_2)$, cleavage of one strand can follow the binding of Mg^{2+} to either E_1 or E_2, and so the apparent K_D will be half of its microscopic

value. In contrast, with $(E_1 \cdot E_2)(P_1 \cdot S_2)$, cleavage of the intact strand requires Mg^{2+} to bind to a specified subunit, so the apparent K_D will be equal to the microscopic constant. On this model, the actual values of the rate constants for cutting the first and second strands of the DNA by *Eco*RV at pH 6.0 may be equal to each other. However, at pH 7.5, where the affinity for Mg^{2+} is much higher (Figure 2A), cleavage of both strands can be coupled, because for the majority of $(E_1 \cdot E_2)(S_1 \cdot S_2)$ complexes Mg^{2+} will be bound to both subunits of the protein.

Single turnovers of the *Eco*RV restriction enzyme were carried out either by mixing one solution of enzyme with another containing both DNA and $MgCl_2$ or by adding $MgCl_2$ to a solution containing both the enzyme and the DNA. No difference at all was observed between these procedures, in both cases yielding data identical with those in Figure 5. This contrasts strongly with the *Eco*RI restriction enzyme where single turnovers starting from the enzyme–DNA complex were markedly different from those starting with the enzyme separate from the DNA (Halford & Johnson, 1983). For the *Eco*RI enzyme, the rate-limiting step appears to be a conformation change of the enzyme–DNA complex that, depending on the nature of the reaction, can affect either one or both subunits of the protein during the lifetime of the enzyme–DNA complex (Halford & Johnson, 1983; Halford, 1983). Hence, the factors that determine whether the *Eco*RI restriction enzyme cleaves one or both strands of the DNA are quite distinct from those shown here for the *Eco*RV enzyme.

In contrast to the above view, derived from the identification of product release as rate limiting for *Eco*RV, Terry et al. (1987) have proposed that the rate-limiting step for *Eco*RI is also its dissociation from the cut DNA. The data of Terry et al. (1987) cannot be compared directly to those of Halford and Johnson (1983), due to different reaction conditions and, more significantly, different DNA substrates, though in neither case can phosphodiester hydrolysis of the enzyme-bound DNA be rate determining. However, a transient phase of product formation prior to the steady state, of the type seen here with *Eco*RV (Figure 4), has never been reported with the *Eco*RI restriction enzyme. Normally, this would indicate that the rate-limiting step for *Eco*RI must precede DNA cleavage but the majority of the *Eco*RI enzyme bound to the DNA may be at nonspecific sequences rather than at the recognition site (Terry et al., 1987), and this would correspondingly reduce the amplitude of the transient phase. The different transient phase and the different single turnover kinetics (where only those for the *Eco*RI enzyme vary between reactions where enzyme was mixed with DNA–Mg^{2+} and where enzyme–DNA was mixed with Mg^{2+}) are both difficult to reconcile with a common process being rate limiting for both *Eco*RI and *Eco*RV.

DNA Supercoiling. The above experiments all used the covalently closed form of pAT153 directly as isolated from *E. coli*. The substrate would therefore be negatively supercoiled with a specific linking difference of about –0.06 (Lilley, 1986a). Negative supercoiling could potentially affect the activity of the *Eco*RV restriction enzyme, as compared to that on relaxed DNA, because it can alter the conformation of the DNA (Lilley, 1986b). In addition, the hydrolysis of a phosphodiester bond in supercoiled DNA liberates more free energy than the same bond in relaxed DNA. To test whether the reactions of *Eco*RV varied with DNA superhelicity, we measured the rates at which this enzyme cleaved covalently closed forms of pAT153 that had either the native level of

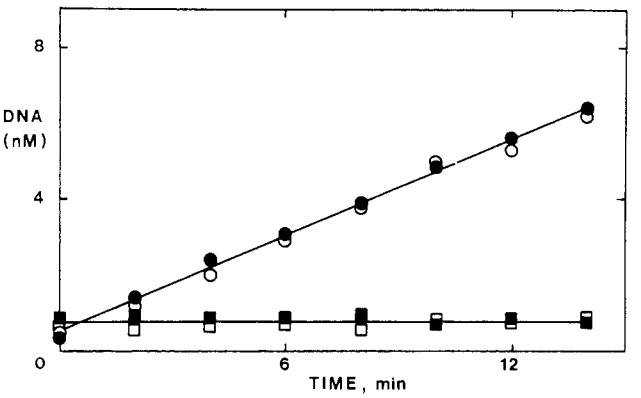

FIGURE 6: Supercoiled and relaxed DNA. Solutions of 10 nM pAT153 (^{3}H-labeled) in 50 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithioerythritol, and 100 μg/mL BSA, pH 7.5, were incubated for 1 h at 37 °C either with (●, ■) or without (○, □) 10 units of topoisomerase I and then heated to 67 °C for 15 min. After the solutions were returned to 37 °C, *Eco*RV restriction endonuclease was added to a final concentration of 0.5 nM. During the reaction with *Eco*RV, samples were removed at timed intervals and analyzed for the amounts of covalently closed, open-circle (■, □), and linear forms (●, ○) of pAT153 (only the latter two are shown).

superhelicity or that had been previously relaxed by treatment of the DNA with topoisomerase I (Figure 6). We also used the restriction endonuclease *Pst*I to linearize pAT153 and then measured the rate at which *Eco*RV cut the linear form into two fragments (data not shown).

Both the relaxed covalently closed DNA and the linear form of pAT153 were cleaved by *Eco*RV at rates that were indistinguishable from those with negatively supercoiled DNA. With the linear substrate, the observed rate corresponds to the cleavage of both strands of the DNA as the products were analyzed by the electrophoresis of duplex DNA; this would not have detected DNA cut in only one strand at the *Eco*RV recognition site. But with the covalently closed form that had been relaxed by topoisomerase I, the reaction of *Eco*RV at pH 7.5 and 10 mM $MgCl_2$ still proceeded directly from covalently closed to linear DNA by cutting both strands; open circles, which would have been produced if the relaxed DNA had been cut initially in one strand of the duplex, were not detected (Figure 6). Hence, as with *Eco*RI (Halford et al., 1979), neither the rates nor the mode of DNA cleavage by the *Eco*RV restriction enzyme varies with the topological state of the DNA. This indicates that the interaction of *Eco*RV with its recognition site does not involve major unwinding of the DNA, such as would be needed to form a cruciform structure (Lilley, 1986b), though kinking of the DNA as seen with *Eco*RI (McClarin et al., 1986) cannot be excluded.

Enzyme Dilutions. Prior to each of the reactions described here, the stock solutions of the *Eco*RV restriction endonuclease were diluted with a buffer that contained BME, BSA, and spermine (Experimental Procedures). The presence of BME in both the enzyme dilution buffer and the reaction mixtures did not alter the activity of the *Eco*RV enzyme; it was included just as a precaution because this enzyme can be inactivated by chemical modification of its single cysteine (Luke et al., 1987). BSA was included because dilute solutions of pure *Eco*RV at <1 μg/mL protein readily lost activity; the addition of BSA helped to stabilize the enzyme, as has been observed with many other restriction endonucleases. However, with *Eco*RV, BSA failed to completely stabilize the dilute enzyme, and spermine (or spermidine) was also needed in the dilution buffer; otherwise, a fraction of the enzyme was inactivated, the fraction varying with the dilution factor (Figure 7). In the absence of spermine, the fraction of *Eco*RV enzyme that

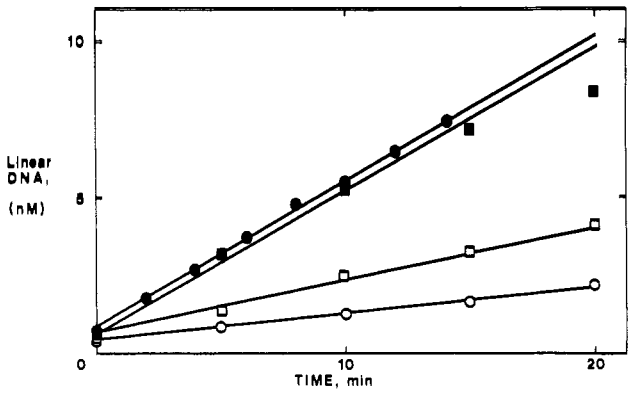

FIGURE 7: Enzyme stability with spermine. *Eco*RV restriction enzyme was diluted with enzyme dilution buffer (Experimental Procedures) that either lacked (○, □) or possessed (●, ■) 1 mM spermine. Reactions at 20 °C were then initiated by adding either 1 μL of 100 nM *Eco*RV (○, ●) or 10 μL of 10 nM *Eco*RV (□, ■), in each case either with or without spermine as indicated, to yield solutions of 200 μL that contained 0.5 nM *Eco*RV restriction endonuclease, 10 nM pAT153 (^{3}H-labeled), and 10 mM $MgCl_2$ in 50 mM Tris, 100 mM NaCl, and 10 mM BME, pH 7.5. For all four reactions, the time course for the production of the linear form of pAT153 is shown.

became inactivated upon dilution was reduced by including the substrate, pAT153, in the dilution buffer (data not shown).

The *Eco*RV enzyme is a basic protein, with a p*I* of 9.4 (Luke et al., 1987), and presumably the effect of the spermine cation is to compete with the nuclease for binding sites on glass or plastic surfaces. DNA could alternatively protect the enzyme from adsorption onto surfaces by forming enzyme–DNA complexes: an effect of this sort might account for some of the kinetic data on the *Hha*II restriction enzyme obtained by Kaddurah-Daouk et al. (1985). We determined that the minimal concentrations needed for full protection of the *Eco*RV enzyme were either 1 mM spermine or 5 mM spermidine. The presence of 1 mM spermine in the enzyme dilution buffer will result in some spermine also being present in the reaction mixture with DNA, where it could affect the activity of *Eco*RV rather than its stability. Certain other restriction enzymes are known to show altered reaction rates in the presence of spermine (Pingoud et al., 1984). We therefore measured rates of DNA cleavage by the *Eco*RV nuclease in reactions identical with those in Figure 3A but with varied spermine concentrations between 10 μM and 10 mM. No variation in reaction rate was seen between 10 μM and 0.5 mM spermine, and only levels above 1.0 mM inhibited the *Eco*RV enzyme (data not shown). Hence, the amount of spermine transferred from the enzyme dilution buffer to the reaction mixtures described here has no effect on the activity of *Eco*RV. The variations of *Eco*RV activity with spermine differ from that of *Eco*RI, which is activated by low concentrations of this ligand (Pingoud et al., 1984).

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