Rapid-Reaction Analysis of Plasmid DNA Cleavage by the *Eco*RV Restriction Endonuclease†

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ABSTRACT: Rapid-reaction methods have been used previously to identify intermediates in the reaction of the *Eco*RV restriction endonuclease on oligonucleotide substrates. In this study, the pathway on macromolecular DNA was elucidated by using the quench-flow method to analyze *Eco*RV reactions on a plasmid with one recognition site. Some reactions were carried out by first allowing the *Eco*RV enzyme to bind nonspecifically to the DNA and then initiating DNA cleavage by adding magnesium ions. The subsequent transfer of the enzyme from nonspecific to specific sites was extremely rapid, at a random walk rate of at least 5×10^5 base pairs per second. The two strands of the DNA at the *Eco*RV recognition site were then cleaved sequentially, at rates that were faster than the turnover number of the enzyme. The rates recorded for the cleavage steps were direct measurements of phosphodiester hydrolysis, while the turnover is limited by the dissociation of the product cleaved in both strands. Other reactions were initiated by adding *Eco*RV and $MgCl_2$ to the DNA: these revealed not only the processes observed in reactions starting from DNA-bound enzyme but also the bimolecular association of the protein with the plasmid. The association rate was limited by diffusion but its rate constant, 1.2×10^8 M^{-1} s^{-1}, was unusually small for the binding of a protein to DNA. The slowness of this diffusion-controlled process may be due to a rapid oscillation of the protein between closed and open conformations, with only the open form capable of binding DNA.

Type II restriction enzymes recognize specific DNA sequences, typically 4−8 bp[1] long, and cleave both strands of the DNA at fixed locations relative to their recognition sites (Roberts & Halford, 1993). To date, over 2800 restriction enzymes have been identified (Roberts & Macellis, 1997), but one of these, the *Eco*RV endonuclease, has been studied in greater depth than perhaps any other (Winkler et al., 1993; Halford et al., 1993; Kostrewa & Winkler, 1995). Despite the lack of sequence homology among the type II endonucleases (Roberts & Halford, 1993), a number of these enzymes, including *Taq*I, *Pvu*II, and *Cfr*10I, show marked similarities to *Eco*RV with respect to their reaction mechanisms and/or three-dimensional structures (Zebala et al., 1992a; Cheng et al., 1994; Bozic et al., 1995). However, some restriction enzymes, such as *Eco*RI and *Bam*HI, differ from *Eco*RV in terms of both mechanism (Terry et al., 1987; Lesser et al., 1993) and structure (Rosenberg, 1991; Newman et al., 1995), though these are similar between themselves (Aggarwal, 1995). Others, such as *Fok*I and *Sfi*I, fall outside both the *Eco*RV and the *Eco*RI groups (Li et al., 1991; Wentzell et al., 1995).

In the presence of Mg^{2+}, the *Eco*RV endonuclease cleaves both DNA strands at the symmetrical sequence, GAT↓ATC (where ↓ marks the point of cleavage: Schildkraut et al., 1984). It exists in solution as a dimer of identical subunits (D'Arcy et al., 1985; Luke et al., 1987). In the crystal structures of *Eco*RV bound to its recognition sequence, the two active sites are juxtaposed against the two scissile phosphodiester bonds in the DNA, one on each strand (Kostrewa & Winkler, 1995). The recognition sequence is cleaved over a million times faster than any other sequence (Taylor & Halford, 1989; Alves et al., 1995). Yet, in binding to DNA in the absence of divalent metal ions, *Eco*RV shows no preference for its recognition site and instead binds all sequences with equal affinity (Taylor et al., 1991; Alves et al., 1995; Szczelkun & Connolly, 1995). This behavior differs from *Eco*RI, which binds its recognition site more tightly than any other sequence (Halford & Johnson, 1980; Jack et al., 1982), but several other restriction enzymes, including *Taq*I and *Cfr*9I, show the same lack of specificity in DNA binding as *Eco*RV (Zebala et al., 1992b; Siksyns & Pleckaityte, 1993). However, in the presence of Ca^{2+}, a mimic for Mg^{2+} which fails to promote DNA cleavage, the *Eco*RV enzyme binds to DNA specifically at its recognition site (Vipond & Halford, 1995). The restraint upon *Eco*RV against cleaving DNA at sites other than its recognition site thus originates from the ternary enzyme−metal−DNA complex rather than the binary enzyme−DNA complex (Halford et al., 1993).

Many of the current perceptions about the mechanism of *Eco*RV stem from its steady-state kinetics with plasmid substrates (Halford et al., 1993). However, steady-state methods provide very little information about intermediates in reaction pathways (Gutfreund, 1995). Moreover, both the

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[1] Abbreviations: bp, base pair(s); E_a, Arrhenius energy; EDTA, ethylenedinitrolotetraacetic acid; k_1 and k_2, rate constants for first and second strand cleavages, respectively; k_a, (pseudo-first-order) association rate constant; k_{ass}, (second-order) association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant (k_d/k_{ass}); RE, restriction endonuclease; $\tau_{1/2}$, reaction half-time.

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DNA recognition and the catalytic functions of *Eco*RV have been extensively analyzed by either mutagenesis of the protein (Vermote et al., 1992; Selent et al., 1992; Vipond & Halford, 1996; Jeltsch et al., 1996) or chemical modification of the DNA (Newman et al., 1990; Hancox et al., 1993; Waters & Connolly, 1994; Thorogood et al., 1996a). The ensuing alterations in enzyme activity were then measured by steady-state methods, which generally failed to reveal which particular step in the mechanism had been affected. To date, intermediates have been detected only in reactions with duplex oligodeoxynucleotides as substrates, which could be monitored by stopped-flow fluorescence and other rapid-reaction methods (Baldwin et al., 1995). But restriction enzymes, like many other DNA-binding proteins, act differently with oligonucleotide substrates compared to macromolecular DNA (Roberts & Halford, 1993). For example, 12 bp substrates for *Eco*RV give much higher values for both k_{cat} and K_m than plasmids (Waters & Connolly, 1994; Baldwin et al., 1995). There are at least two reasons why oligonucleotides should behave differently from DNA macromolecules. One is that the energies for the electrostatic interactions between protein and DNA will vary considerably between oligomeric and polymeric substrates, because only the latter permit counterion condensation (Zhang et al., 1996). Another is that binding of a protein to a specific site on macromolecular DNA can involve an initial interaction at any location on the DNA followed by an intramolecular transfer to the specific site, perhaps by linear diffusion along the DNA (Berg et al., 1982). The dissociation of the protein from a specific site on macromolecular DNA can also occur via nonspecific sites (Jack et al., 1982).

The reactions of the *Eco*RV endonuclease on macromolecular substrates thus need to be analyzed by transient kinetics. However, reactions with natural plasmids are limited to nanomolar concentrations, and this precludes the use of stopped-flow fluorescence and most other optical techniques, since these generally require micromolar concentrations.[2] An alternative approach to transient kinetics is the quench-flow method, as used previously on *Eco*RI (Terry et al., 1987). In this study, a microvolume quench-flow technique (Eccleston et al., 1985) was used to measure the fast kinetics for single turnovers of *Eco*RV on a plasmid substrate. The following paper (Hancox & Halford, 1997) describes an application of the kinetic methods developed here to mutationally altered *Eco*RV proteins.

EXPERIMENTAL PROCEDURES

Reagents. RE buffer is 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 100 μg/mL bovine serum albumin. *Eco*RV restriction endonuclease was purified as before (Luke et al., 1987). Its concentration was determined by absorbance at 280 nm (D'Arcy et al., 1985) and is given in terms of the dimeric protein, M_r 5.7×10^4. The monomeric form of the plasmid pAT153 was labeled with [*methyl*-^{3}H]thymidine and purified as described previously (Halford & Goodall, 1988). DNA concentrations were determined by absorbance at 260 nm and are given in terms of the 3658 bp molecule, M_r 2.36×10^6. Analysis of the plasmid preparations by agarose gel electrophoresis (Halford & Goodall, 1988) showed that, typically, 90% of the DNA was covalently closed supercoiled material and 10% nicked open-circles. In all figures shown here that depict the production of open-circle DNA during the time course of *Eco*RV reactions, the observed amount of this form has been corrected for the fraction present at zero time.

Quench-Flow. The RQF-63 apparatus (Hi-Tech Scientific Ltd., Salisbury, U.K.) was used to mix two solutions, 80 μL from each, to give reactions containing *Eco*RV endonuclease, pAT153 (^{3}H-labeled), and $MgCl_2$ in RE buffer. [All reactant concentrations given below refer to those after mixing equal volumes of the two solutions.] After the requisite time delay, the solution of mixed reactants was added to the quench solution, 80 μL of 100 mM EDTA in 50 mM Tris-HCl, pH 8.0. The time delay was varied from 5 ms (the minimum for this device) to 10 s. Typically, each reaction was repeated two to three times at each time point (the error bars on the data points in the figures shown below denote the range of values from these repeats) and with a total of 15−20 different time points per reaction. Prior to and during the reactions, the two reagent solutions and the quench solution were held in the thermostated bath of the RQF-63 at the requisite temperature. Following the addition of the quench, the RQF-63 delivers the central portion of the quenched solution (~200 μL) to an external loop. The sample was recovered from this loop and the DNA rescued by precipitation with ethanol. The DNA was resuspended in Tris-EDTA and subjected to electrophoresis through agarose under conditions where the supercoiled, open-circle, and linear forms of pAT153 are separated from each other (Halford & Goodall, 1988). Slices of agarose encompassing each form of the DNA were excised from the gel and analyzed by scintillation counting (Taylor & Halford, 1989).

In control experiments, 0.1 M NaOH was used as the quench instead of EDTA, but this yielded the same kinetics for *Eco*RV as those which used EDTA as the quench (data not shown). The chelation of the Mg^{2+} required for DNA cleavage by *Eco*RV thus appears to halt the reaction instantaneously. Other tests examined whether supercoiled pAT153 suffered any shear damage when driven through this rapid mixing device at its maximal flow rate, 8.85 mL/s: no such damage was detected (data not shown). The validity of the time base from this quench-flow apparatus was checked by using it to measure rates for the alkaline hydrolysis of 2,4-dinitrophenyl acetate in parallel with stopped-flow experiments where the same reaction was monitored by the increase in absorbance at 365 nm (Barman & Gutfreund, 1964): in all cases, the rate constants measured by quench-flow were the same (±10%) as those from stopped-flow.

Curve Fitting. For reactions of the type A → B and A → B → C, first-order rate constants were evaluated from the experimental data by nonlinear least squares regression to the appropriate analytical equations (Gutfreund, 1995) using GRAFIT (Erithacus Software, Slough, U.K.). For consecutive reactions, the rate constant for the A → B stage was first determined from the exponential decline of [A] with time and this value was then used as an initial estimate in fitting the time dependence of either [B] or [C] to the rate

[2] A stopped-flow fluorescence method had been used to monitor plasmid DNA cleavage by the *Eco*RI endonuclease (Halford & Johnson, 1983): reactions were carried out in the presence of ethidium bromide and monitored from the increase in fluorescence due to the open-circle and linear DNA products binding more ethidium than the supercoiled substrate, though this additional binding only occurred upon the relaxation of the supercoils after the dissociation of the DNA from the protein. However, ethidium is a more potent inhibitor of *Eco*RV than *Eco*RI, so this assay cannot be applied to *Eco*RV (Luke et al., 1987).

constants for both the A → B and the B → C stages. Standard deviations were usually assessed from three or more repeats for each experiment. GRAFIT was also used for fitting data to linear dependencies and for hyperbolic functions. For mechanisms involving three or more rate constants, the individual rate constants were evaluated from sets of experimental data by regression analysis to the numerical solutions for the differential equations defining the reaction mechanism, the solutions being obtained by using SCIENTIST (MicroMath Software, Salt Lake City, UT). The algorithm from Badcoe (1992) for multiple binding of proteins to macromolecular DNA was used as before (Taylor et al., 1991).

RESULTS

Experimental Strategy. The plasmid pAT153 is isolated from *recA* strains of *Escherichia coli* as a covalently closed circle of supercoiled DNA containing one copy of the recognition sequence for *Eco*RV amid 3658 bp. The reaction of the *Eco*RV endonuclease at its recognition site on this plasmid can be detected readily: the cleavage of one strand results in the conversion of the supercoiled DNA to its open-circle form while the cleavage of both strands generates the linear form. In standard reaction buffers, DNA cleavages at all other sites occur at least a million times more slowly and are not detected (Taylor & Halford, 1989 & 1992). The supercoiled, open-circle, and linear forms can be separated from each other by electrophoresis, and, by using DNA labeled with [^{3}H]thymidine, the amounts of each of these three forms, sampled at each time point during the reaction, can be measured by scintillation counting. This assay has been used before for the steady-state kinetics of *Eco*RV reactions (Halford & Goodall, 1988; Vipond et al., 1995) and in the comparisons of wild-type and mutant *Eco*RV proteins (Vermote et al., 1992; Vipond et al., 1996). Under steady-state conditions, with a lower concentration of enzyme than pAT153, and in the optimal reaction buffer for *Eco*RV, the plasmid was cleaved directly to linear DNA, without liberating any of the open-circle form. Hence, each turnover results in the cleavage of both strands before the dissociation of the final product. Steady-state reactions on pAT153 yielded values of 0.5 nM for K_m and 0.015 s^{-1} for k_{cat} (Halford & Goodall, 1988; Taylor & Halford, 1989).

One way to measure the rates for the DNA cleavage steps in the catalytic cycle of *Eco*RV, as opposed to the steady-state rate for the complete cycle, is by carrying out single-turnover reactions, with the enzyme in molar excess of the plasmid. The k_{cat} for *Eco*RV on pAT153 noted above corresponds to a $\tau_{1/2}$ of 45 s for the complete cycle, so it might have been possible to perform single-turnover reactions on *Eco*RV by hand-mixing the reagents. However, when excess *Eco*RV was added by hand to the pAT153 and the reaction sampled at 10 s intervals thereafter, the rates for product formation could only be measured in reactions employing suboptimal conditions for *Eco*RV: under optimal conditions, all of the DNA was cleaved within the first 10 s (Halford & Goodall, 1988). A rapid-reaction technique was therefore required to monitor single turnovers of *Eco*RV. The quench-flow method proved amenable (Figure 1).

Mixing Procedures. In some cases, the quench-flow apparatus was used to mix one solution containing a high concentration of the *Eco*RV endonuclease with a second solution containing a lower concentration of pAT153, with either one or both solutions also containing the Mg^{2+} ions necessary for DNA cleavage: in these instances, reactions at the same final concentrations of reagents yielded the same rates regardless of whether the $MgCl_2$ was placed in the enzyme solution or the DNA solution, or both (data not shown). At varied times after mixing enzyme and DNA, in the range from 5 ms to 10 s, the reaction was quenched by the addition of EDTA and the DNA was recovered for

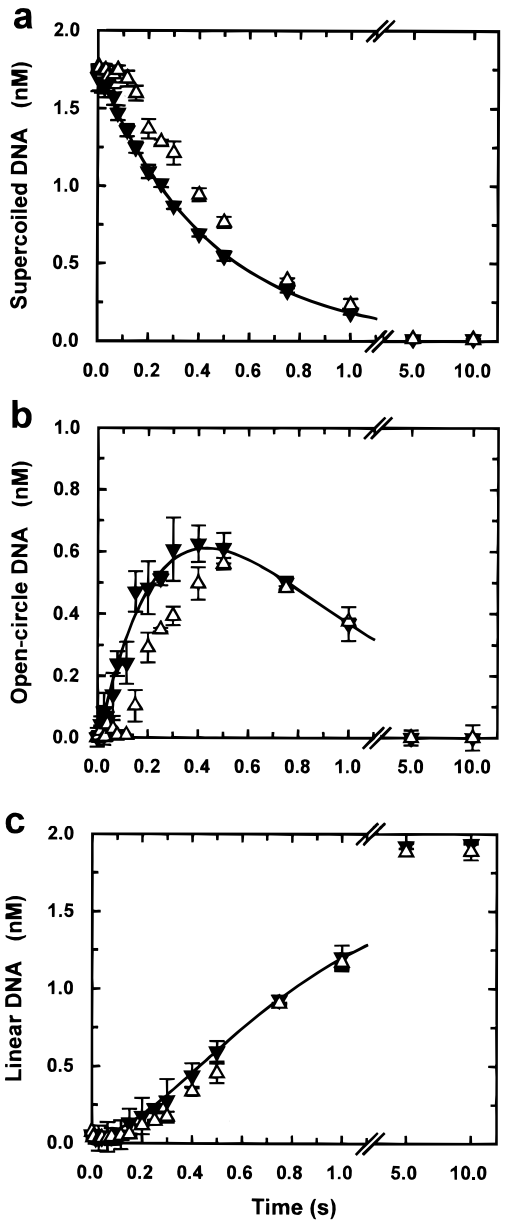

FIGURE 1: Quench-flow analysis. The reactions contained 35 nM *Eco*RV endonuclease, 2 nM pAT153 (90% supercoiled DNA), and 10 mM $MgCl_2$ in RE buffer at 25 °C. After the time intervals indicated on the *x*-axes, the reactions were quenched by the addition of a one-half volume of 0.1 M EDTA and the amounts of the supercoiled, open-circle, and linear forms of pAT153 were determined as in Experimental Procedures (panels a, b, and c, respectively). In all three panels, unfilled data points (△) refer to reactions in which the above composition was obtained by mixing together one solution containing *Eco*RV enzyme and $MgCl_2$ with a second solution containing the DNA; filled data points (▼) refer to reactions in which the same composition was obtained by mixing together one solution containing the *Eco*RV enzyme and the DNA with a second solution containing the $MgCl_2$. The lines drawn through the filled data points are the theoretical lines for S_B (panel a), O_B (panel b), and L_B (panel c) from eq 1, calculated from the values for k_1 and k_2 that yield the optimal fit to the experimental data, 2.9 and 3.2 s^{-1}, respectively.

subsequent analysis by agarose gel electrophoresis. The amounts of the supercoiled, open-circle, and linear forms were then evaluated at each time point tested during the reaction (Figures 1a, 1b, and 1c, respectively: unfilled data points). In other cases, noted below as premix reactions, the *Eco*RV endonuclease was first added to the pAT153 in a buffer that lacked divalent metal ions, and this solution was then mixed in the quench-flow instrument with one that contained $MgCl_2$. At varied times after adding the Mg^{2+} ions to the enzyme−DNA solution, these reactions were likewise quenched with EDTA and the amounts of substrate and products measured as above (Figure 1: filled data points).

In the premix reactions, a decrease in the amount of the supercoiled form of pAT153 was observed at the earliest time points tested, and this decrease continued progressively with increasing reaction times: the complete progress curve for the amount of supercoiled DNA against time followed a single exponential all the way through from the start to the finish of the reaction (Figure 1a). The premix reactions also resulted in open-circle DNA being formed from the beginning of the reaction (Figure 1b), while essentially none of the linear DNA was produced straightaway. Instead, linear DNA production commenced after an initial lag phase (Figure 1c). At longer time points, the subsequent increase in the amount of linear DNA paralleled the decrease in the amount of open-circle DNA, in the manner expected for a sequential reaction where the DNA is first nicked in one strand and then in the other. However, when the reactions were initiated by mixing one solution of pAT153 with another containing both *Eco*RV and $MgCl_2$, the amounts of supercoiled, open-circle, and linear DNA observed at the early time points deviated from their counterparts in the premix reactions. The decline in the amount of supercoiled DNA with time no longer followed a single exponential but instead went through a lag phase before any reaction occurred (Figure 1a). An equivalent lag phase also preceded the formation of open-circle DNA, whereas open-circle production had started from the beginning of premix reactions (Figure 1b). Finally, the lag phase characteristic of a sequential reaction seen prior to linear DNA formation in the premix reactions was now stretched out over a longer time base (Figure 1c).

In the reactions starting from enzyme and DNA in separate solutions, the enzyme that cleaves the DNA must be in free solution at the time of mixing the reagents. The observed differences between this mixing protocol and the premix reactions can only be due to the latter being caused by enzyme already bound to the DNA at the start of the reaction. The following analysis considers first the reactions using the premix procedure and subsequently the reactions starting with enzyme separate from the DNA.

Premix Reactions. In the absence of divalent metal ions, the *Eco*RV endonuclease binds to DNA in a completely nonspecific manner, with no preference for its recognition site over any other DNA sequence (Taylor et al., 1991; Alves et al., 1995; Szczelkun & Connolly, 1995). Since the binding of one molecule of *Eco*RV covers about 15 bp of DNA (Winkler et al., 1993), a 3658 bp DNA such as pAT153 could carry ~245 molecules of *Eco*RV at the same time, though this potential will seldom be realized on account of the steric exclusion between overlapping sites and between sites that are separated by less than the size of the protein (McGhee & von Hippel, 1974). Nevertheless, given the molar excess of *Eco*RV over pAT153 used here, the premix reactions will start with the plasmid already bound by several molecules of the enzyme though it is unlikely that any of these will be located at the recognition site before the addition of Mg^{2+}. The mode for the number of *Eco*RV molecules bound to pAT153 at any particular values for both protein and DNA concentrations (Table 1) can be calculated by coupling the known K_D value for the binding of *Eco*RV to any individual site on the DNA (0.9 μM for nonspecific binding in the absence of divalent metal ions; Taylor et al., 1991; Vipond & Halford, 1995) to the algorithm of Badcoe (1992) for the packing of objects onto an extended matrix (I. G. Badcoe, personal communication).[3] If it is then assumed that the bound molecules of *Eco*RV are distributed randomly along the DNA, the mode of the distance between the *Eco*RV recognition site and the nearest molecule of *Eco*RV protein can also be calculated (Table 1).[3]

Table 1: Pre-Mix Reactions at Varied Concentrations of *Eco*RV[a]

*Eco*RV (nM)	mol of *Eco*RV bound per mol of pAT153	distance from *Eco*RV site to nearest protein (bp)	k_1 (s^{-1})	k_2 (s^{-1})
35	15	114	2.9 ± 0.06	3.2 ± 0.2
100	43	42	3.4 ± 0.2	2.8 ± 0.2
400	126	14	3.8 ± 0.2	4.1 ± 0.8

[a] Given the concentration of *Eco*RV endonuclease shown in the left-hand column, in a binding mix with 2 nM pAT153 in RE buffer, the mode of the number of molecules of *Eco*RV bound to each molecule of pAT153 was calculated as described in the text, as was the mode of the distance (in bp) between the *Eco*RV recognition site on pAT153 and the nearest molecule of *Eco*RV. The latter calculation assumed that the *Eco*RV endonuclease was bound to pAT153 in the absence of Mg^{2+} at random positions throughout its length. To evaluate k_1 and k_2, quench-flow experiments were carried out by mixing one solution containing the *Eco*RV endonuclease and pAT153 with another containing $MgCl_2$, to give reactions in RE buffer at 25 °C with 2 nM pAT153, 10 mM $MgCl_2$ and the indicated concentration of *Eco*RV endonuclease. The concentrations of the supercoiled, open-circle, and linear forms of pAT153 were measured at time points during the reactions and these readings were fitted to the appropriate derivations from eq 1 to find the values for k_1 and k_2 that gave the minimal least-squares deviations from the experimental data.

The concentrations of the supercoiled [S], open-circle [O], and linear [L] forms of pAT153 measured at each time point during the premix reactions were found to fit closely (Figure 1) to those expected for the reaction scheme

$$S_B \xrightarrow{k_1} O_B \xrightarrow{k_2} L_B \qquad (1)$$

where k_1 and k_2 are the rate constants for first and second strand cleavages and where the subscript B denotes that all three forms of the DNA are enzyme-bound. The question

[3] When several molecules of protein are bound to each molecule of DNA, some DNA molecules will have bound n molecules of protein; others will have $n+1$, $n+2$, ..., $n+i$ molecules, following a Gaussian distribution. The number of bound proteins cited here is the mode (i.e., the most populated state amid the distribution) rather than the mean since the latter will generally have a non-integral value and is thus meaningless. Similarly, the statistical expectations for the distance between the *Eco*RV recognition site and the nearest *Eco*RV protein are given in terms of modal values, that for the most populated species in the distribution of multiply-bound DNA molecules. However, the assumption that the *Eco*RV enzyme will be distributed randomly along the DNA is an oversimplification. Nonspecific binding by *Eco*RV shows some cooperativity, with successive binding events occurring preferentially to adjacent sites (Taylor et al., 1991). The values cited in Table 1 for the modes of the distance between recognition site and protein are thus likely to be underestimates of the true distances.

now arises as to whether k_1 and k_2 are the true rate constants for phosphodiester hydrolysis in each strand of the DNA. An alternative possibility is that even though the conversion of S_B to O_B requires enzyme at the recognition site on pAT153, S_B could still refer to an initial complex at any location on the plasmid and that the rates being measured here are limited by the transfer of the protein from nonspecific to specific sites. The experiments in this study measure only DNA cleavage at the recognition site rather than binding to this site.

If increasing amounts of *Eco*RV endonuclease are added to a fixed amount of pAT153, the number of molecules of *Eco*RV bound to each molecule of DNA will rise, thus reducing the distance along the DNA between the recognition site and the nearest molecule of *Eco*RV (Table 1). If the transfer from nonspecific to specific sites occurs by linear diffusion, then the time for the transfer should show a square-power dependence on the length of DNA traversed (Berg et al., 1982). To examine this possibility, quench-flow experiments employing the premix strategy were carried out at a fixed concentration of pAT153 but with higher enzyme concentrations than that used for Figure 1. As before, the amounts of the supercoiled, open-circle, and linear forms of pAT153 were measured at successive time points and the data (not shown) were fitted to eq 1 in order to determine values for k_1 and k_2 (Table 1). In contrast to expectation, the value for k_1 showed only a marginal increase as the concentration of *Eco*RV was increased, even when raised by enough to give an 8-fold reduction in the length of DNA that the protein would need to travel to the recognition site. The values for k_2 showed no systematic dependence on the concentration of *Eco*RV protein (Table 1). Hence, the rate of DNA cleavage at the *Eco*RV recognition site, by an enzyme molecule initially located on the DNA ~114 bp away from the recognition site, cannot be limited by the transfer time between nonspecific and specific sites.

Additional quench-flow experiments were carried out with a modification of the premix procedure, where $CaCl_2$ was added to either the solution of $MgCl_2$ or to both the enzyme−DNA and $MgCl_2$ solutions (Figure 2). Ca^{2+} acts as a mimic for Mg^{2+} in promoting the specific binding of *Eco*RV to its recognition site but, instead of allowing the DNA cleavage reaction, it acts as an inhibitor of the Mg^{2+}-dependent reaction (Vipond & Halford, 1995; Vipond et al., 1995). The premix reaction with Ca^{2+} in the enzyme−DNA solution should thus start with the enzyme located at the recognition site while the premix without Ca^{2+} in the enzyme−DNA solution starts with the enzyme at nonspecific sites, so the former will yield a faster rate than the latter whenever the transfer between nonspecific and specific sites becomes rate-limiting. However, both types of reactions with Ca^{2+} present might yield lower rates than the equivalent reaction lacking Ca^{2+}, on account of its inhibition of phosphodiester hydrolysis. The rate at which *Eco*RV cleaved its recognition site on pAT153 was indeed lower in the presence of Ca^{2+} (Figure 2) compared to the equivalent reaction without Ca^{2+} (Figure 1a), 0.7 s^{-1} instead of 2.9 s^{-1}, but the same rates were obtained regardless of whether Ca^{2+} was present in the enzyme−DNA mix. Thus the transfer from nonspecific to specific sites rate must be faster than the rate of DNA cleavage observed in the presence of Ca^{2+}.

Reactions from Separate Solutions. The reactions of the *Eco*RV endonuclease on pAT153 were analyzed further by using the quench-flow device to mix enzyme in one solution with DNA in a separate solution, with $MgCl_2$ in either the enzyme or DNA solutions or both. These were carried out under single-turnover conditions, with the enzyme in molar excess over the plasmid, and the extent of DNA cleavage was again determined by measuring the amounts of the supercoiled [S], open-circle [O], and linear [L] DNA at each time point. However, in contrast to the reactions initiated by adding Mg^{2+} to a solution containing both enzyme and DNA, where the time course for the decline in [S] followed a single exponential, the reactions starting from enzyme and DNA in separate solutions showed a preliminary lag phase before any of the supercoiled substrate was converted to open-circle DNA (Figure 1a).

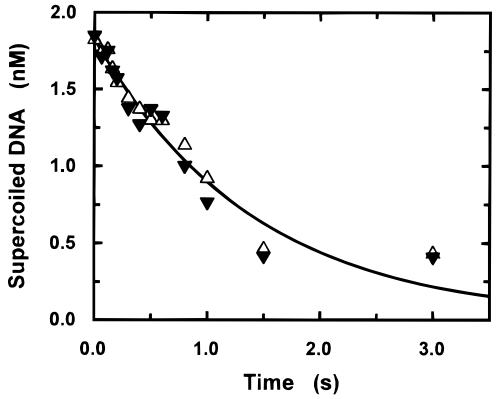

FIGURE 2: Effect of Ca^{2+} ions on premix reactions. Both reactions contained 35 nM *Eco*RV endonuclease, 2 nM pAT153, 10 mM $MgCl_2$, and 0.25 mM $CaCl_2$ at 25 °C. In one case (unfilled data points, △), the reactions were set up by mixing one solution of *Eco*RV endonuclease and pAT153 in RE buffer with a second solution containing $MgCl_2$ and $CaCl_2$ (at 0.5 mM) in RE buffer. In the other case (filled data points, ▼), the reactions were set up by using the quench-flow apparatus to mix one solution of *Eco*RV endonuclease, pAT153, and $CaCl_2$ (at 0.25 mM) in RE buffer with a second solution of $MgCl_2$ and $CaCl_2$ (at 0.25 mM) in RE buffer. The reactions were allowed to proceed for the time intervals shown on the *x*-axis before being stopped with EDTA: the amount of the supercoiled form of pAT153 left at each time point tested was then measured. The line drawn for both data sets is the optimal fit to a single-exponential, yielding $k_1 = 0.71 \pm 0.08$ s^{-1}.

The simplest explanation for this lag phase is that it reflects the initial binding of *Eco*RV to its recognition site on the plasmid. If so, the mechanism in eq 1 can be modified to

$$S_F \underset{k_d}{\overset{k_a}{\rightleftarrows}} S_B \xrightarrow{k_1} O_B \xrightarrow{k_2} L_B \qquad (2)$$

where S_F refers to the free plasmid, k_a the pseudo-first-order rate for the bimolecular association and k_d that for the dissociation of the protein from the recognition site. All other terms are as in eq 1, with the conversion of S_B to O_B once again requiring *Eco*RV at the recognition site even though the route to this complex may involve nonspecific complexes elsewhere on the DNA. For a reaction initiated by adding enzyme to DNA, the DNA starts in its free state, without any enzyme bound, so the experimental values for [S] that were measured at each time point during the reaction now correspond to $[S_F + S_B]$ instead of $[S_B]$ alone. The lag phase preceding the decline in $[S_F + S_B]$ is then a consequence of the consecutive steps in this reaction pathway. The decline in the concentration of $S_F + S_B$ with time is equivalent to that for the increase in the final product from a two-step consecutive reaction, of the type A → B → C, where the increase in [C] will display a detectable lag

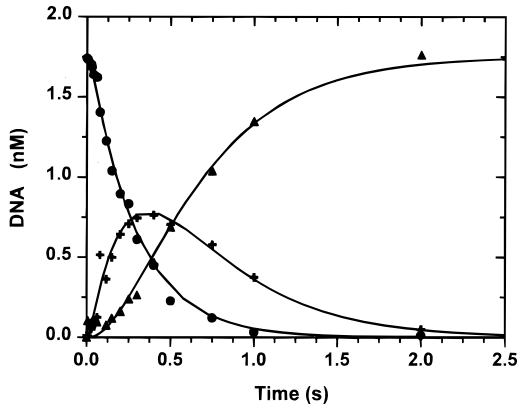

FIGURE 3: Fitted curves to reactions from separate enzyme and DNA solutions. The quench-flow apparatus was used to mix one solution containing *Eco*RV endonuclease and $MgCl_2$ with a second solution containing pAT153, to yield a reaction with 100 nM *Eco*RV endonuclease, 2 nM pAT153, and 10 mM $MgCl_2$ in RE buffer at 25 °C. The reaction proceeded for the time intervals shown on the *x*-axis before being stopped with EDTA: the amount of the supercoiled (●), open-circle (+), and linear (▲) forms of pAT153 at each time point tested was then measured. The lines drawn are the theoretical lines from eq 2 for $[S_F + S_B]$, $[O_B]$, and $[L_B]$, respectively, with the values for k_a, k_d, k_1, and k_2 that give the optimal fit to the experimental data. The values were $k_a = 47\ s^{-1}$; $k_d = 1.0\ s^{-1}$; $k_1 = 3.8\ s^{-1}$; and $k_2 = 2.7\ s^{-1}$.

phase whenever the two rate constants are within an order of magnitude of each other (Fersht, 1985; Gutfreund, 1995).

Attempts were made to evaluate all four rate constants in eq 2 by an iterative fitting routine (see Experimental Procedures) that started by taking a set of values for the rate constants and then calculating values for $[S_F + S_B]$, $[O_B]$, and $[L_B]$ at successive time points during the reaction: the calculated values were compared with the experimental values for [S], [O], and [L] and the cycle was then repeated over and over again with new sets of numbers for the four rate constants until the fit between calculated and experimental data could be improved no further. Unfortunately, this routine yielded ill-conditioned fits in that several alternative sets of numbers for the four rate constants gave equally good matches to the experimental data. However, by holding k_1 and k_2 to the values obtained from premix reactions (Table 1), the routine produced well-defined values as initial estimates for k_a and k_d. Subsequent rounds of iteration in which the values for k_a, k_d, k_1, and k_2 were all allowed to vary from these estimates gave an optimal fit with a single set of numbers for the four rate constants (Figure 3).

To test whether eq 2 was an adequate description of the reactions starting from *Eco*RV and pAT153 in separate solutions, the analysis was carried out across a range of enzyme concentrations. Single-turnover conditions were maintained throughout the range: the lowest concentration of *Eco*RV enzyme still exceeded the concentration of pAT153. At each enzyme concentration, the changes in the amounts of the supercoiled, open-circle, and linear forms of pAT153 that were observed during the reactions followed the same general pattern as that shown in Figure 3. In each case, values for k_a, k_d, k_1, and k_2 were determined by the fitting routine described above (Figure 4). If eq 2 is an adequate description, then kinetic theory (Gutfreund, 1995) predicts that the values for the four rate constants ought to vary with the enzyme concentration, $[E_0]$, as follows: the values for k_a should increase linearly with increasing $[E_0]$; those for k_1 should vary with $[E_0]$ in hyperbolic fashion; those for both k_2 and k_d should be independent of $[E_0]$. In all four cases, the ways in which the parameters varied with $[E_0]$ matched these expectations (Figure 4).

The gradient for the linear increase in the values for k_a, the pseudo-first-order constants for the binding of *Eco*RV to pAT153, gave a second-order rate constant for the association, k_{ass}, at $1.2 \times 10^8\ M^{-1}\ s^{-1}$ (Figure 4a). Although the values for k_d determined by the routine described above were somewhat scattered (Figure 4b), due to the curve-fitting being comparatively insensitive to the precise value for k_d, the mean value yielded a dissociation rate constant of *Eco*RV from this substrate at $1.2\ s^{-1}$. The ratio of these numbers for k_d and k_{ass} gives an equilibrium dissociation constant (K_D) of 10 nM for the $S_F \leftrightarrow S_B$ equilibration, the binding of *Eco*RV to its recognition site on pAT153 under these particular reaction conditions (10 mM $MgCl_2$, 100 mM NaCl). Since this equilibration determines the degree to which the substrate is saturated with enzyme, it accounts for why the rate (k_1) at which *Eco*RV cleaves the first DNA strand should show a hyperbolic dependence on $[E_0]$. The hyperbolic relationship between the values for k_1 and $[E_0]$ (Figure 4c) yielded a figure for K_D at 14 nM, in amazingly good agreement with that from the k_d/k_{ass} ratio. The $S_F \leftrightarrow S_B$ equilibration will not affect the rate (k_2) at which *Eco*RV cuts the second DNA strand since cleavage of the first strand is effectively an irreversible step.

Single-turnover reactions starting with the *Eco*RV endonuclease separate from the pAT153 were also examined at various temperatures between 15 and 35 °C, while maintaining fixed concentrations of endonuclease, plasmid, and $MgCl_2$. At each temperature, the changes in the amounts of the supercoiled, open-circle and linear forms of pAT153 during the reactions again followed the same general pattern as that in Figure 3. The progress curves were analyzed as above to evaluate k_a, k_1, and k_2 (eq 2) at each temperature and the temperature dependencies for these rate constants displayed as Arrhenius plots (Figure 5). All three rate constants gave linear Arrhenius plots over this temperature range, but the plot for k_a had a shallower gradient than those for either k_1 and k_2, the latter pair having indistinguishable slopes. The values for k_a increased slightly with increasing temperature and yielded an Arrhenius energy (E_a) of only 3.2 kcal/mol. In contrast, the values for k_1 and k_2 both increased steeply with increasing temperature and yielded the same value for E_a at 24.4 kcal/mol (Figure 5).

DISCUSSION

In vivo, restriction endonucleases will only encounter macromolecular DNA substrates where the recognition site(s) are embedded in an array of alternative sequences (Roberts & Halford, 1993). Their function in vivo is thus fundamentally different from that seen in vitro with short DNA substrates, made from synthetic oligonucleotides, that carry essentially nothing but the recognition sequence. In vitro reactions with macromolecular substrates, such as phage DNA or plasmids, provide a better analogy for their activities in vivo. Nevertheless, on account of the unwieldiness of plasmids relative to synthetic oligonucleotides, most of the current information about individual steps in the reaction pathways for restriction enzymes has come from studies with oligonucleotide substrates (Lesser et al., 1993; Baldwin et al., 1995; Cao et al., 1995). The purpose of this study was to analyze the separate steps in the pathway for *Eco*RV on

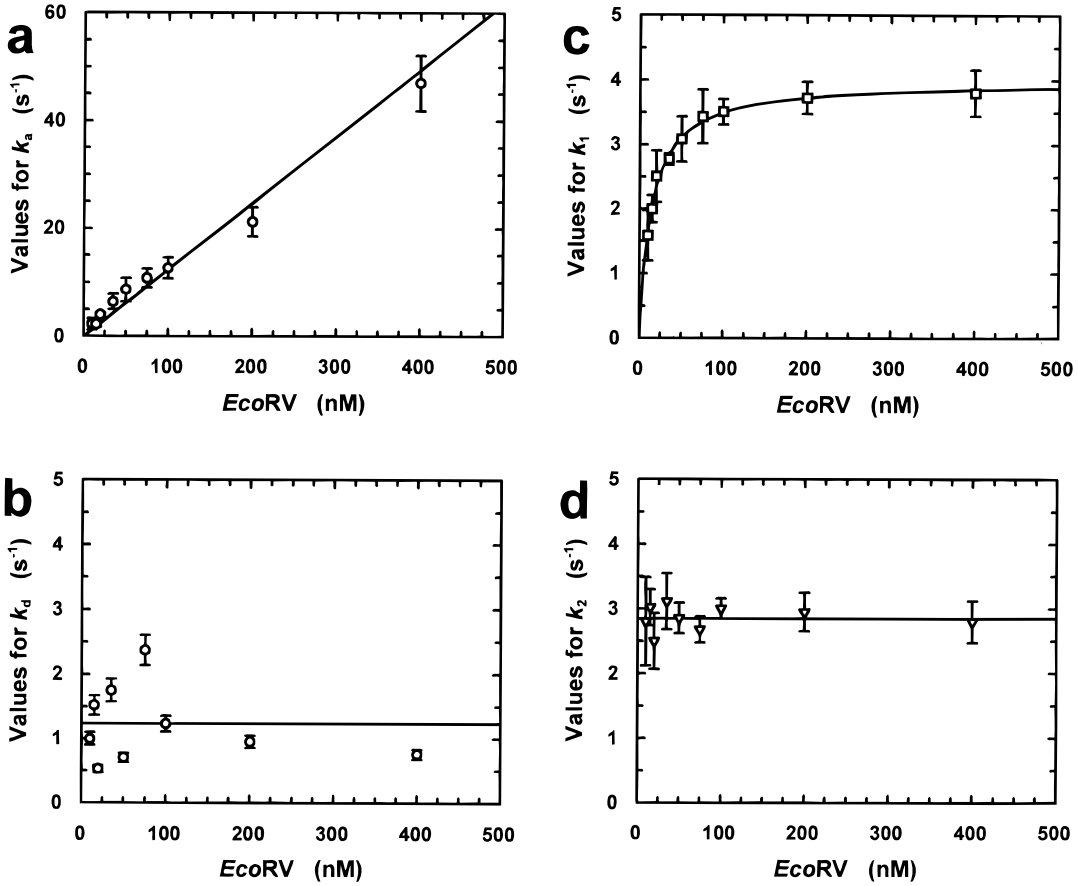

FIGURE 4: Concentration dependencies for reactions from separate enzyme and DNA solutions. Following the mixing of one solution containing *Eco*RV endonuclease and $MgCl_2$ with a second solution containing pAT153, the reaction possessed the concentration of *Eco*RV endonuclease indicated on the *x*-axes with 2 nM pAT153 and 10 mM $MgCl_2$ in RE buffer at 25 °C. The reactions at each enzyme concentration were analyzed as in Figure 3 to yield values for k_a (panel a), k_d (panel b), k_1 (panel c), and k_2 (panel d). The standard deviations from the fitting routine are marked by error bars. The lines drawn are by regression to the theoretical patterns from eq 2 for how these rate constants should vary with the concentration of *Eco*RV endonuclease: for panel a, a linear increase in the values for k_a with increasing [*Eco*RV] [fitted optimally with a gradient of 1.2 ($\pm$0.2) $\times$ 10^8 M^{-1} s^{-1}]; for panels b and d, constancy in the values for both k_d or k_2 with increasing [*Eco*RV] (the lines drawn are parallel to the *x*-axes and yield means of 1.2 $\pm$ 0.6 s^{-1} for k_d and 2.8 $\pm$ 0.2 s^{-1} for k_2); for panel c, a hyperbolic increase in the values for k_1 with increasing [*Eco*RV] (fitted optimally with K_D = 1.4 ($\pm$0.09) $\times$ 10^{-8} M and k_1 = 4.0 $\pm$ 0.06 s^{-1} at infinite [*Eco*RV]).

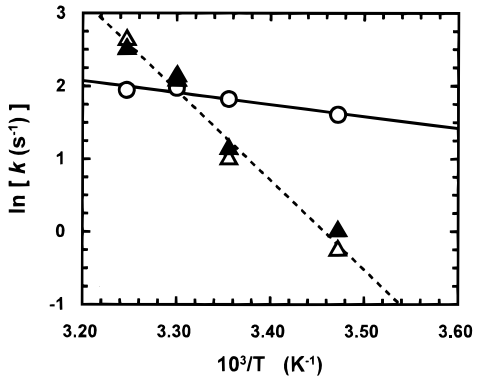

FIGURE 5: Temperature dependencies for reactions from separate enzyme and DNA solutions. Following the mixing of one solution containing *Eco*RV endonuclease and $MgCl_2$ with a second solution containing pAT153, the reactions were with 35 nM *Eco*RV endonuclease, 2 nM pAT153 and 10 mM $MgCl_2$ in RE buffer, at varied temperatures in the range from 15, to 35 °C. The reactions at each temperature were analyzed as in Figure 3 to obtain values for k_a (○), k_1 (△), and k_2 (▲). The resultant relationships between reaction temperature and the values for k_a, k_1 and k_2 are plotted in Arrhenius format with one solid line for the k_a values (fitted to E_a = 3.2 $\pm$ 0.7 kcal/mol) and one dashed line for both k_1 and k_2 values (fitted to E_a = 24.4 $\pm$ 2.1 kcal/mol).

a macromolecular substrate with one recognition site. Under the same reaction conditions as used here, the steady-state kinetics for *Eco*RV on pAT153 yields a k_{cat} of 0.015 s^{-1} (Halford & Goodall, 1988) while the k_{cat} with a 12 bp duplex is almost 50 times higher, at 0.7 s^{-1} (Waters & Connolly, 1994; Baldwin et al., 1995). However, steady-state kinetics cannot reveal which particular step(s) in the pathway differ sufficiently between oligomeric and polymeric substrates to explain this large difference in k_{cat}.

DNA Cleavage. The rapid-reaction method used here yielded the rates at which *Eco*RV cleaves both first and second strands of its recognition site on pAT153. At saturating concentrations of the endonuclease, the rate constant for cutting the first strand (k_1) is about 4 s^{-1} while that for the second (k_2) is about 3 s^{-1}, regardless of whether the reactions start from enzyme and DNA in the same solution (Table 1) or enzyme and DNA in separate solutions (Figures 3 and 4). However, supercoiled pAT153 can be converted to open-circles by cutting either the "top" or "bottom" strands of the DNA while the conversion from open-circles to linear DNA can only occur by cutting one particular strand, whichever had not been cleaved in the first reaction. In order to compare the rates for phosphodiester hydrolysis on the intact substrate with that on the nicked DNA, the apparent rate constant for cutting the first strand

thus needs to be divided by a factor of 2 (Halford & Johnson, 1983; Zebala et al., 1992b). With this correction, the rate for cutting the first strand is, if anything, slightly smaller than that for the second strand though the two constants are clearly very similar to each other.

The rates recorded for the DNA cleavage reactions are not necessarily limited by the chemical events and might instead be determined by an earlier step in the reaction pathway. Perhaps the chemical step is preceded by a conformational change in either the protein or the DNA, or both, that limits the rate at which the DNA is cleaved. The conformation of the *Eco*RV protein undergoes extensive alterations as it binds to nonspecific DNA and is altered further upon binding to specific DNA (Winkler et al., 1993). The structure of the specific DNA also undergoes major adjustments as it interacts with the protein (Kostrewa & Winkler, 1995; Vipond & Halford, 1995; Thorogood et al., 1996b). These changes might occur as the dimeric enzyme interacts with the intact duplex before cutting either DNA strand. But a slow isomerization at this stage would result in the rate of open-circle formation being smaller than that for its utilisation, in contradiction to the observations reported here (Table 1, Figure 4). Alternatively, the conformational changes needed to accommodate the nicked DNA left after cutting one strand might differ from those required for the initial substrate. But the possibility of two separate rates being determined by different conformational changes is difficult to reconcile to the identical Arrhenius energies for first and second strand cleavages (Figure 5). The activation energy barrier for the process that limits the rate of first strand cleavage is the same as that independently limiting the rate for second strand cleavage. The barrier is likely to be determined by the transition state for the chemical reaction, the pentacoordinated phosphoryl group at the scissile bond, since this is common to both reactions, while a common barrier for two different conformational changes is an unlikely event. The structural changes observed by X-ray crystallography (Winkler et al., 1993) seem to occur too rapidly to limit the rates for DNA cleavage, as was also found in stopped-flow studies on oligonucleotide cleavage (Baldwin et al., 1995).

The rate constants for the DNA cleavage steps thus appear to be determined by the chemical reactions. These rates are similar to that measured by rapid reaction methods for the DNA cleavage step during the reaction of *Eco*RV on a 12 bp substrate under comparable conditions, 2.5 s^{-1} (Baldwin et al., 1995). In all probability, the rate constants for phosphodiester hydrolysis by *Eco*RV are the same on macromolecular and oligonucleotide substrates. These values cannot account for the 50-fold difference in k_{cat}.

Product Dissociation. For the reaction on the 12 bp duplex, the rate constant for the DNA cleavage step is within an order of magnitude of the k_{cat} and independent measurements of the rate constant for product dissociation showed that k_{cat} was partly determined by the DNA cleavage step and partly by product dissociation (Baldwin et al., 1995). In contrast, the DNA cleavage steps in the reaction on pAT153 are about 200 times faster than the k_{cat}, so the rate-limiting step for the complete catalytic cycle must occur after the cleavage reactions. These results support the proposal made previously (Halford & Goodall, 1988), that the k_{cat} for *Eco*RV on plasmid substrates is limited by the dissociation of the final product cleaved at the recognition site in both strands. They also show that the different kinetics with oligomeric and polymeric substrates for *Eco*RV are due primarily to different rates for product dissociation, that for the oligomer being much faster. This difference could be due to the *Eco*RV enzyme departing from the cleaved recognition site on macromolecular DNA by first transferring to nonspecific DNA before dissociating into free solution, as suggested for the *Eco*RI endonuclease (Jack et al., 1982), while the departure from a cleaved oligonucleotide must occur directly into free solution.

The location of the rate-limiting step at the end of the pathway accounts for the divergence of the K_D and K_m values for the interaction of *Eco*RV with its recognition site on pAT153. The K_D was evaluated at 10 nM from the ratio of the dissociation and association rate constants, k_d and k_{ass} respectively (Figures 4a and 4b), and at 14 nM from the hyperbolic dependence for k_2 (Figure 4c): the K_m was previously evaluated at 0.5 nM (Taylor & Halford, 1989). However, for a mechanism of the type

$$E + S \leftrightarrow ES \rightarrow EP \rightarrow E + P \qquad (3)$$

the K_m can only equal the K_D when the ES $\rightarrow$ EP step is rate-limiting (Fersht, 1985). Whenever the EP $\rightarrow$ E + P step is rate-limiting, the value for K_m must be smaller than that for the K_D (Gutfreund, 1995). Under the reaction conditions used here, the K_D for the binding of *Eco*RV to its recognition site on pAT153, 10 or 14 nM, is smaller than the K_D for the binding of *Eco*RV to any DNA sequence in the absence of divalent metal ions (0.9 μM: Taylor et al., 1991), but it is not as small as the K_D for *Eco*RV binding to its recognition site in the presence of Ca^{2+} (0.2 nM: Vipond & Halford, 1995). By extrapolation from Jencks (1975), the instability of the *Eco*RV−metal−DNA complex with Mg^{2+}, relative to that with Ca^{2+}, presumably reflects the need for the complex with the active cofactor to be at a higher energy level than the complex with the inactive cofactor: the active complex should be closer to the transition state of the reaction than the inactive complex.

The fact that the DNA cleavage steps in the reaction of *Eco*RV on pAT153 are about 200 times faster than k_{cat} demonstrates the inappropriateness of steady-state kinetics in assessing the effects of mutations in the protein or chemical modifications to the DNA. For instance, if an amino acid substitution causes a 10-fold reduction in k_{cat}, it might be concluded that the amino acid in question plays only a minor role in the activity of the enzyme. But a 10-fold reduction in k_{cat} could be due to a 2000-fold reduction in the rate for the DNA cleavage step. Such a mutant of *Eco*RV is described in the following paper (Hancox & Halford, 1997). Other mutations in *Eco*RV have been characterized previously where it was thought that the effect on k_{cat} was too small for that amino acid to have any direct role in catalysis, but these could also turn out to have large effects on the DNA cleavage rate. The mutations at Glu45 might fall into this category since these possess low levels of activity, as opposed to zero activity (Selent et al., 1992), while other studies suggest that Glu45 plays a key role in catalysis by *Eco*RV (Kostrewa & Winkler, 1995; Vipond et al., 1995; Bozic et al., 1996).

DNA Binding. The quench-flow experiments starting with enzyme and DNA in separate solutions yielded rate constants for the binding and dissociation of *Eco*RV from pAT153 (Figures 4a and 4b). Since these rate constants were determined from their influence on the subsequent DNA

cleavage reactions at the recognition site, they refer to the rates at which *Eco*RV binds to (or dissociates from) this particular site on pAT153. This is likely to be a two-step process in which the enzyme binds first to a nonspecific site anywhere on the plasmid and then moves to the recognition site (Taylor et al., 1991; Jeltsch et al., 1996). Intramolecular transfer of *Eco*RV from nonspecific to specific sites had been demonstrated previously by adding to a mixture of labeled DNA and *Eco*RV a solution containing both $MgCl_2$ and a large excess of unlabeled DNA: the labeled DNA was cleaved preferentially (Taylor et al., 1991). The quench-flow experiments in this study show that the transfer is a rapid process, much faster than any of the other processes being measured here. When Ca^{2+} was used to position the *Eco*RV enzyme at its recognition site on the DNA (Vipond & Halford, 1995), the subsequent DNA cleavage rate was the same as in the control with *Eco*RV bound initially to nonspecific sites (Figure 2). Moreover, loading the DNA in the absence of Mg^{2+} with increasing amounts of *Eco*RV, so that the distance along the DNA between the recognition site and the nearest molecule of *Eco*RV was progressively reduced, had no significant effect upon the rate of the cleavage reaction (Table 1). An effect due to the distance between the recognition site and the protein might have been observed at low concentrations of *Eco*RV, since these would have compelled the protein to travel greater distances to the recognition site. For example, at 35 nM *Eco*RV, the mode of the distance between the recognition site and the nearest molecule of *Eco*RV is 114 bp (Table 1), but this is increased to 366 bp at 10 nM *Eco*RV.[3] However, such experiments would have required *Eco*RV concentrations below the K_D (14 nM: Figure 4c) for its binding to the recognition site, conditions where it would have been impossible for the reaction rates not to vary with the enzyme concentration.

The rate for the association of *Eco*RV with its recognition site is thus limited by its initial binding to nonspecific DNA and the second-order rate constant measured here, 1.2×10^8 M^{-1} s^{-1} (Figure 4a), reflects its association with the entire DNA molecule. Many proteins bind their substrates at rates of $\sim 1 \times 10^8$ M^{-1} s^{-1} (Fersht, 1985) but rates for DNA−protein associations are generally faster than this, often $>1 \times 10^9$ M^{-1} s^{-1} (Berg et al., 1982). The comparatively low rate for the binding of *Eco*RV to DNA is not a consequence of the quench-flow method used here. Similar experiments on the binding of Tn*21* resolvase to its target sites on a plasmid yielded an association rate constant of 1×10^9 M^{-1} s^{-1} (Oram et al., 1997). Yet the association of *Eco*RV with pAT153 is still likely to be limited by diffusion. In a diffusion-controlled reaction, every collision between the reactants possesses sufficient energy to complete the reaction. Such reactions always display low Arrhenius energies, typically <5 kcal/mol (Gutfreund, 1995). The Arrhenius energy for this binding reaction, 3.2 kcal/mol (Figure 5), falls within the range for diffusion-controlled events and is low enough to suggest that the *Eco*RV endonuclease encounters no significant activation energy barriers on its route from free solution to its recognition site on pAT153.

The apparent dichotomy between the diffusion-controlled nature of this reaction and its comparatively slow rate can be explained by reference to the crystal structures of *Eco*RV (Winkler et al., 1993). In the DNA−protein complex, the DNA is located in a deep cleft between the protein subunits but, in the structure of the free protein, the cleft is too narrow to accommodate DNA. DNA cannot be docked into the structure of the free protein so as to leave it in the position it occupies in the DNA−protein complex, without first widening the inter-subunit cleft. The free protein may therefore oscillate between the structure seen by X-ray crystallography, with a closed cleft, and another state where the cleft is open enough to bind DNA. If the equilibrium constant between closed and open states was 0.1, for example, then only 10% of the protein would be competent for DNA binding at any one time. Under the reaction conditions used here, with the enzyme in excess over the DNA, the pseudo-first-order rate constant for the binding (k_a) is related to the second-order rate constant by the expression $k_a = k_{ass}[E_0]$. Hence, if the effective value for E_0 is one-tenth of the total concentration of enzyme, then the value for k_{ass} would then be 10 times higher than the estimate of 1.2×10^8 M^{-1} s^{-1} given here. However, the oscillation between closed and open conformations would have to be faster than the binding reaction. If the oscillation limited the binding rate, then the values for k_a would not show their linear increase with increasing $[E_0]$ (Figure 4a) but would instead be invariant with $[E_0]$.

The transfer of the *Eco*RV endonuclease from a nonspecific site on pAT153 to its recognition site is likely to occur by linear diffusion along the DNA, of the sort discussed by Berg et al. (1982). The movement of proteins along DNA has been analyzed with both the *Eco*RI and *Eco*RV endonucleases by determining how the interaction varies with the length of the DNA (Jack et al., 1982; Ebrecht et al., 1985; Terry et al., 1987; Jeltsch et al., 1994, 1996). These studies have yielded estimates for the random walk rate of the protein on the DNA, in the range from 10^5 to 10^7 bp/s. The data in Table 1 permit the setting of a lower limit for the random walk rate by *Eco*RV, from the observation that the endonuclease can traverse at least 114 bp of DNA in a time that is short enough to have no influence on the subsequent DNA cleavage reaction. To have no influence, the transfer time must be ~10 times shorter than the $\tau_{1/2}$ of the reaction, the latter being 250 ms (Figure 1a). Given the dead time of 5 ms for the quench-flow apparatus, a 25 ms delay before DNA cleavage in the premix reactions would have been detected readily. If the 114 bp are covered by a random walk that has equal probabilities for steps in forward and reverse direction, then the overall distance covered will be $(114)^2$ bp in <25 ms, which corresponds to $>5 \times 10^5$ bp/s. This lower limit falls within the range of estimates for random walk rates noted above, though only the estimates in the upper end of the range are consistent with the speed of site location by *Eco*RV.

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REFERENCES

Aggarwal, A. K. (1995) *Curr. Opin. Struct. Biol. 5*, 11−19.
Alves, J., Selent, U., & Wolfes, H. (1995) *Biochemistry 34*, 11191−11197.
Badcoe, I. G. (1992) *Comput. Appl. Biosci. 8*, 323−330.
Baldwin, G. S., Vipond, I. B., & Halford, S. E. (1995) *Biochemistry 34*, 705−714.
Barman, T. E., & Gutfreund, H. (1964) in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Eisenhardt, R., Gibson, Q., & Lonberg-Holm, K., Eds.) pp 339−344, Academic Press, New York.

Berg, O. G., Winter, R. B., & von Hippel, P. H. (1982) *Trends Biochem. Sci. 7*, 52−55.
Bozic, D., Grazulis, S., Siksnys, V., & Huber, R. (1996) *J. Mol. Biol. 255*, 176−186.
Cao, W., Mayer, A. N., & Barany, F. (1995) *Biochemistry 34*, 2276−2283.
Cheng, X., Balendiran, K., Schildkraut, I., & Anderson, J. (1995) *EMBO J. 13*, 3927−3935.
D'Arcy, A., Brown, R. S., Zabeau, M., van Resandt, R. W., & Winkler, F. K. (1985) *J. Biol. Chem. 260*, 1987−1990.
Ebrecht, H. J., Pingoud, A., Urbanke, C., Maass, G., & Gualerzi, C. (1985) *J. Biol. Chem. 260*, 6160−6166.
Eccleston, J. F., Dix, D. B., & Thompson, R. C. (1985) *J. Biol. Chem. 260*, 16237−16241.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman, New York.
Gutfreund, H. (1995) *Kinetics for the Life Sciences*, Cambridge University Press, Cambridge, U.K.
Halford, S. E., & Johnson, N. P. (1980) *Biochem. J. 191*, 593−604.
Halford, S. E., & Johnson, N. P. (1983) *Biochem. J. 211*, 405−415.
Halford, S. E., & Goodall, A. J. (1988) *Biochemistry 27*, 1771−1777.
Halford, S. E., Taylor, J. D., Vermote, C. L. M., & Vipond, I. B. (1993) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Vol. 7, pp 47−69, Springer-Verlag, Berlin, Germany.
Hancox, E. L., Connolly, B. A., & Walker, R. T. (1993) *Nucleic Acids Res. 21*, 3485−3491.
Hancox, E. L., & Halford, S. E. (1997) *Biochemistry 36*, 7577−7585.
Jack, W. E., Terry, B. J., & Modrich, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4010−4014.
Jeltsch, A., Alves, J., Wolfes, H., Maass, G., & Pingoud, A. (1994) *Biochemistry 33*, 10215−10219.
Jeltsch, A., Wenz, C., Stahl, F., & Pingoud, A. (1996) *EMBO J. 15*, 5104−5111.
Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219−410.
Kostrewa, D., & Winkler, F. K. (1995) *Biochemistry 34*, 683−696.
Lesser, D. R., Kurpiewski, M. R., Waters, T., Connolly, B. A., & Jen-Jacobson, L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7548−7522.
Li, L., Wu, L., & Chandrasegaran, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4275−4279.
Luke, P. A., McCallum, S. A., & Halford, S. E. (1987) *Gene Amplif. Anal. 5*, 183−205.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469−489.
Newman, P. C., Williams, D. M., Cosstick, R., Seela, F., & Connolly, B. A. (1990) *Biochemistry 29*, 9902−9910.
Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., & Aggarwal, A. K. (1995) *Science 269*, 656−663.
Oram, M., Marko, J. F., & Halford, S. E. (1997) *J. Mol. Biol.* (in press).
Roberts, R. J., & Halford, S. E. (1993) in *Nucleases*, 2nd ed. (Linn, S. M., Lloyd, R. S., & Roberts, R. J., Eds.) pp 35−88, Cold Spring Harbor Laboratory Press, Plainview, NY.
Roberts, R. J., & Macelis, D. (1997) *Nucleic Acids Res. 25*, 248−262.
Rosenberg, J. M. (1991) *Curr. Opin. Struct. Biol. 1*, 104−113.
Schildkraut, I., Banner, C. D. B., Rhodes, C. S., & Parekh, S. (1984) *Gene 27*, 327−329.
Selent, U., Ruter, T., Köhler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschläger, T., Wolfes, H., Peters, F., & Pingoud, A. (1992) *Biochemistry 31*, 4808−4815.
Siksnys, V., & Pleckaityte, M. (1993) *Eur. J. Biochem. 217*, 411−419.
Szczelkun, M. D., & Connolly, B. A. (1995) *Biochemistry 34*, 10724−10733.
Taylor, J. D., & Halford, S. E. (1989) *Biochemistry 28*, 6198−6207.
Taylor, J. D., & Halford, S. E. (1992) *Biochemistry 31*, 90−97.
Taylor, J. D., Badcoe, I. G., Clarke, A. R., & Halford, S. E. (1991) *Biochemistry 30*, 8743−8753.
Terry, B. J., Jack, W. E., & Modrich, P. (1987) *Gene Amplif. Anal. 5*, 104−118.
Thorogood, H., Grasby, J. A., & Connolly, B. A. (1996a) *J. Biol. Chem. 271*, 8855−8862.
Thorogood, H., Waters, T. R., Parker, A. W., Wharton, C. W., & Connolly, B. A. (1996b) *Biochemistry 35*, 8723−8733.
Vermote, C. L. M., Vipond, I. B., & Halford, S. E. (1992) *Biochemistry 31*, 6089−6097.
Vipond, I. B., & Halford S. E. (1995) *Biochemistry 34*, 1113−1119.
Vipond, I. B., & Halford S. E. (1996) *Biochemistry 35*, 1701−1711.
Vipond, I. B., Baldwin G. S., & Halford S. E. (1995) *Biochemistry 34*, 697−704.
Waters, T. R., & Connolly, B. A. (1995) *Biochemistry 34*, 1812−1819.
Wentzell, L. M., Nobbs, T. J., & Halford, S. E. (1995) *J. Mol. Biol. 248*, 581−595.
Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K., & Wilson, K. S. (1993) *EMBO J. 12*, 1781−17945.
Zebala, J. F., Choi, J., Trainor, J., & Barany, F. (1992a) *J. Biol. Chem. 267*, 8106−8116.
Zebala, J. F., Choi, J., & Barany, F. (1992b) *J. Biol. Chem. 267*, 8097−8105.
Zhang, W., Bond, J. P., Anderson, C. F., Lohman, T. M., & Record, M. T. Jr. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2511−2516.

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