Steady-state parameters governing cleavage of pBR322 DNA by EcoRI endonuclease are highly sensitive to ionic environment, with $K_m$ and $k_{cat}$ increasing 1,000-fold and 15-fold, respectively, when ionic strength is increased from 0.059 to 0.23 M. By contrast, pre-steady-state analysis has shown that recognition, as well as first and second strand cleavage events that occur once the enzyme has arrived at the EcoRI site, are essentially insensitive to ionic strength, and has demonstrated that the rate-limiting step for endonuclease turnover occurs after double-strand cleavage under all conditions tested. Furthermore, processive cleavage of a pBR322 variant bearing two closely spaced EcoRI sites is governed by the same turnover number as hydrolysis of parental pBR322, which contains only a single EcoRI sequence, ruling out slow release of the enzyme from the cleaved site or a slow conformational change subsequent to double-strand cleavage. We attribute the effects of ionic strength on steady-state parameters to nonspecific endonuclease-DNA interactions, reflecting facilitated diffusion processes, that occur prior to EcoRI sequence recognition and subsequent to DNA cleavage.

EcoRI endonuclease is among the simplest of the site-specific DNA enzymes and has proven useful for study of the mechanisms governing the interaction of such proteins with DNA (1–4). The enzyme functions as a homodimer of a 31-kDa polypeptide (5–9), and in presence of Mg$^{2+}$ introduces two staggered, single-strand scissions into the symmetric recognition sequence, 5'GAATTCC-3' (10). In the absence of a divalent cation, the endonuclease binds specifically and with high affinity to its recognition sequence (7, 11–13).

Work from several laboratories has indicated that DNA cleavage by EcoRI endonuclease proceeds by the mechanism shown in Fig. 1, with double-strand cleavage proceeding via an intermediate species (E1) containing one single-strand break within the EcoRI sequence (5, 14, 15). The fate of E1 is determined by reaction conditions and the nature of the substrate: the intermediate dissociates from the enzyme in the case of some DNAs but not others (5, 14, 16–18). Chemical quench experiments have indicated that product release is rate-limiting for turnover on CoEl and pBR322 plasmid DNAs under one set of experimental conditions (5, 15), and facilitated diffusion has been implicated in the paths by which the endonuclease locates an EcoRI sequence and deparst from a cleaved site (19–22). However, the effects of reaction parameters that are expected to affect the efficiency of facilitated diffusion have not been examined with respect to catalytic behavior of the enzyme.

We have therefore systematically analyzed the kinetics of the endonuclease using steady-state and pre-steady-state methods. This work demonstrates that product release is rate-limiting for turnover at ionic strengths of 0.06–0.23 M and show that, although variation of ionic strength has dramatic effects on $K_m$ and $k_{cat}$, the kinetics of events that occur at the recognition sequence are essentially insensitive to variation of this parameter. We also show that the protein remains associated with the polynucleotide product after cleavage, undergoing facilitated transfer between DNA sites so rapidly that the $k_{cat}$ for processive cleavage of a two-site substrate is the same as that for cleavage of an otherwise identical DNA containing a single EcoRI site. These observations indicate that the steady-state behavior of the enzyme on natural substrates is dominated by nonspecific interactions, reflecting the significance of facilitated diffusion processes in the reaction mechanism.

Materials and Methods

Enzymes and DNA—Homogeneous EcoRI endonuclease was prepared as described previously (23). Other restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Covalently closed, circular pBR322 and pBR322(RI), (Fig. 2; see Ref. 20) were isolated by published methods (5, 24). PvuII-linearized pBR322 was prepared as 5'-end-labeled as described previously (15, 19). Nick translation was used to prepare [32P]pBR322(RI). Plasmid DNA was treated with 35 units of Clal endonuclease at 16 °C in 0.02 M Tris-HCl (pH 7.6), 0.02 M NaCl, 6 mM MgCl$_2$, 1 mM dithiothreitol, 0.10 mg/ml bovine serum albumin, and 75 μg/ml ethidium bromide for 30 min to produce molecules bearing a single-strand break at either of the two Clal sites present on the plasmid. Final DNA preparations contained 75–80% open circles, 20–23% covalently closed circles, and 2–4% linear molecules. These preparations were radiolabeled by nick translation in the presence of [α-32P]dTTP (25), followed by closure of the single-strand break with T4 DNA ligase. Nucleic acid pellets recovered after phenol extraction and ethanol precipitation were dried briefly in vacuo and resuspended in 0.1 ml of 0.02 M Tris-HCl (pH 7.6), 0.05 M NaCl, 1 mM EDTA. DNA was separated from unincorporated radiolabel by gel filtration through Sephacryl S-300 equilibrated in 0.02 M Tris-HCl (pH 7.6), 0.05 M NaCl, 1 mM EDTA. Two to 10 mol of radiolabeled dTMP were incorporated per mol of plasmid DNA, and ligation efficiencies were 88–92%. To prepare radiolabeled linear pBR322(RI) with both EcoRI sites located near one end (Fig. 2), nick-translated [32P]pBR322(RI) DNA was digested with HindIII endonuclease according to the manufacturer's instructions. Linear plasmid DNA was recovered after phenol extraction and ethanol precipitation as described above.

Steady-state Cleavage Initiated by Endonuclease Addition to Solutions Containing DNA and Mg$^{2+}$—Reactions under standard EcoRI cleavage conditions were performed at 37 °C in 0.1 M Tris-HCl (pH 7.6), 0.05 M NaCl, 5 mM MgCl$_2$, 0.2 mM EDTA, 0.05 mM bovine serum albumin, and DNA as indicated. Effects of NaCl on cleavage were determined in reactions containing 0.02 M Tris-HCl (pH 7.6), 5 mM MgCl$_2$, 0.2 mM EDTA, 0.05 mg/ml bovine serum albumin, 0.025–0.20 mM NaCl, and 5'-end-labeled DNA as indicated. Cleavage was initiated by addition of 0.05 volume of diluent (5) containing an appropriate
least squares regression analysis (27) using the integrated rate equations for the mechanism shown in Reaction 1 as described previously (15). Values for \( k_i \) were obtained from fitting data for disappearance of substrate to an exponential decay mechanism. These \( k_i \) values were used as initial estimates in fitting data for the open circular intermediate and the linear DNA product. The reported values for \( k_i \) were obtained from analysis of the appearance of the double-strand cleaved product and did not deviate from those obtained by analysis of the formation and decay of the open circular intermediate by more than 10%.

Pre-steady-state Cleavage Initiated by Mixing DNA with Solutions Containing EcoRI Endonuclease—Pre-steady-state experiments were also performed by mixing an endonuclease-MgCl\(_2\) solution with \(^{3}H\)pBR322. Solutions containing 0.02 M Tris-HCl (pH 7.6), 0.2 mM EDTA, 0.05 mg/ml bovine serum albumin, 0.025–0.20 mM NaCl, and 2–20 mM \(^{3}H\)pBR322 were prewarmed to 37 °C, after which DNA cleavage was initiated by mixing samples (40 \( \mu \)l) with an equal volume of a solution containing 0.01 mM MgCl\(_2\) and EcoRI endonuclease in the same buffer. The reported values for \( k_i \) were obtained from analysis of the appearance of the double-strand cleaved product and did not deviate from those obtained by analysis of the formation and decay of the open circular intermediate by more than 10%.

FIG. 1. Kinetic mechanism for EcoRI endonuclease. S, 1, and 2 represent DNA where the EcoRI site is intact (S), cleaved in one strand (1), or cleaved in both strands (2). Panel A, facilitated diffusion has been implicated in the kinetic paths by which the endonuclease locates an intact EcoRI sequence and and leaves the cleaved site under some reaction conditions (19, 20). This effect is indicated by (\( E \))\(_N\), which represents the population of non-specific complexes involved in this phenomenon. Rate constants \( k_i \) and \( k_j \) govern the fate of the E-1 intermediate, which can dissociate from the endonuclease surface in the case of some DNA substrates (5, 14, 16–18). Panel B, since the non-specific complexes implicated in EcoRI endonuclease catalysis cannot be directly detected during analysis of cleavage kinetics, the mechanism has been simplified for kinetic studies. Since dissociation of the E-1 intermediate is negligible with the pBR322 substrate used in this study (16, 18, 29), this step can be neglected in the experiments described here.

amount of EcoRI endonuclease. Reactions were terminated by addition of 0.2 vol 50% (v/v) glycerol, 1% sodium dodecyl sulfate, 0.05 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol, and products were resolved by electrophoresis through 1% agarose gels in a Tris borate buffer system. Bands were visualized by ethidium fluorescence or by autoradiography, excised, and DNA products quantified by liquid scintillation counting. Steady-state Cleavage Initiated by MgCl\(_2\) Addition to Previously Formed Endonuclease-DNA Complexes—Reactions containing 0.02 mM Tris-HCl (pH 7.6), 0.02 mM EDTA, 0.05 mg/ml bovine serum albumin, 5\( \mu \)M end-labeled DNA, EcoRI endonuclease, and NaCl as indicated were incubated at 37 °C until equilibrium was attained (7). Cleavage was initiated by adding reaction buffer containing MgCl\(_2\) and a 17-fold molar excess of unlabeled pBR322 DNA (relative to \(^{3}H\)DNA) to yield a final MgCl\(_2\) concentration of 5 mM. Reactions were terminated and products quantified as described above.

Pre-steady-state Cleavage Initiated by MgCl\(_2\) with Specific EcoRI Endonuclease-DNA Complexes—Pre-steady-state chemical quench experiments with previously formed endonuclease-DNA complexes were performed by a modification of the previously described procedure (15) using a RQF-3 quench flow apparatus (KinTek Instruments). Reactant loops and mixing block were maintained at 37 °C by circulating water. Calibration was verified weekly by performing KOH-catalyzed hydrolysis of p-nitrophenyl acetate at 25 °C (second order rate constant \( k_{\text{off}} \) = 570 M\(^{-1}\) s\(^{-1}\) ) (26). The value for \( k_{\text{off}} \) determined with the KinTek instrument was 538 ± 30 M\(^{-1}\) s\(^{-1}\) (one standard deviation, \( n = 12 \)).

Specific EcoRI-DNA complexes were formed by incubating endonuclease and \(^{3}H\)pBR322 at 37 °C in 0.02 mM Tris-HCl (pH 7.6), 0.025–0.2 mM NaCl, 0.2 mM EDTA, and 0.05 mg/ml bovine serum albumin. DNA cleavage was initiated by mixing samples (40 \( \mu \)l) with an equal volume of 0.01 mM MgCl\(_2\) in the same buffer. The final concentration of \(^{3}H\)pBR322 was 1–10 mM, and final endonuclease concentrations varied between 20 and 200 nM, with higher concentrations of the two components being required to drive specific complex formation at higher ionic strengths. The component concentrations used in each experiment were based on equilibrium affinity constants determined under identical conditions (7) and ensured that >97% of the plasmid DNA was bound specifically by the enzyme.

Reactions were quenched by mixing with 0.5 volumes of 0.075 M EDTA. Collected samples also contained an additional 140 \( \mu \)l of 0.075 M EDTA delivered by the quench flow apparatur during sample ejection. Quenched samples were collected in 1.5-ml micro centrifuge tubes containing 20 \( \mu \)l of 10% sodium dodecyl sulfate and were stored on ice until all time points had been collected. Samples were dried in vacuo, resuspended in 75 \( \mu \)l of 0.1 mM Tris-HCl (pH 7.6), 0.05% bromphenol blue, 5% glycerol (v/v), and reaction products were quantified after agarose gel electrophoresis as described above. Rate constants governing first (\( k_i \)) and second (\( k_j \)) strand cleavage events were estimated by nonlinear
processive events and substrate concentration, considering the effects of ionic strength at the extremes of are dependent on substrate concentration. This can be seen by site; $E$ fragment containing the pairs) is a derivative of pBR322 with a duplication of a 192-bp $Bsp$ end. Cleavage of a single $Eco$ site produces either 4477- and 80-bp fragments or 4528- and 29-bp DNA fragments. Cleavage of both sites produces 4477-, 51-, and 29-bp DNA fragments.

is limited to a maximum of 0.5 on linear substrates (20). Values for $f_0$ were calculated as described by Terry et al. (20), using the following equation,

$$f_0 = V_{51}/(V_{51} + V_{29}),$$

where $f_0$ is the fraction of cleaved molecules that have participated in processive events and $V_{51}, V_{29},$ and $V_{50}$ are the initial rates of formation of the 51-, 80-, and 29-bp cleavage products.

RESULTS

Dependence of Steady-state Kinetic Parameters on Ionic Strength—The equilibrium affinity of $Eco$ RI endonuclease for its recognition sequence (7, 13), kinetic parameters governing formation and dissociation of specific complexes (19, 28), and the effective length of the DNA segment scanned by positionally correlated facilitated diffusion (20, 29) are highly dependent on ionic strength. Fig. 3 demonstrates that salt concentration also has a dramatic effect on the steady-state parameters that govern cleavage of pBR322 DNA. An increase in the NaCl concentration from 0.025 to 0.2 M (ionic strength of 0.050–0.23 M) results in a 1,000-fold increase in the $K_m$ for pBR322 and an increase in $k_{cat}$ of about 15-fold. The endonuclease is thus unusual in the sense that optimal ionic conditions for cleavage are dependent on substrate concentration. This can be seen by considering the effects of ionic strength at the extremes of substrate concentration, i.e., [DNA] → 0 and [DNA] → $\infty$. At dilute DNA concentrations, where velocity is proportional to the $k_{cat}/K_m$ ratio, the rate of cleavage will be highest at low salt concentration because $K_m$ decreases more rapidly with decreasing ionic strength than does $k_{cat}$. However, at high DNA concentration where rate is $k_{cat}$-limited, the cleavage rate will be highest at high ionic strength because $k_{cat}$ increases with salt concentration. The significance of these effects in terms of mechanism will be considered below.

Rate Constants for Sequence Recognition and Strand Cleavage Steps Are Independent of Ionic Strength—For purposes of pre-steady-state analysis, the mechanism shown in Fig. 1 may be simplified to that shown in Reaction 1.

This simplification is valid for pBR322 because the $E$-1 intermediate does not dissociate with this DNA (16, 18, 29). A previous chemical quench flow analysis has indicated that this scheme is an adequate representation of the mechanism (15). Since this previous study was restricted to analysis of the fate of preformed $E$/$S$ complexes at a single ionic strength, we have extended this analysis to a range of NaCl concentrations using two mixed-endonuclease preparations. In the first, preformed endonuclease-pBR322 complexes were mixed with a MgCl$_2$ solution, whereas in the alternate protocol DNA was mixed with a solution of endonuclease and MgCl$_2$. The former method bypasses steps occurring prior to specific complex formation, whereas the rate of first strand cleavage in the latter protocol can potentially be limited by enzyme-DNA association, or by other slow steps on the path to specific complex formation.

Fig. 4 shows an example of pre-steady-state cleavage of endonuclease-pBR322 complexes formed prior to initiation of DNA cleavage by addition of MgCl$_2$. The fit of the data to a two-step mechanism is excellent, with the only notable deviation occurring late in the reaction and reflecting persistence of a small amount of the open circular intermediate species.$^2$ We conclude that double-strand cleavage occurring within previously formed site-specific complexes can be described by the

$^2$At high ratios of endonuclease to DNA, we have observed the accumulation of DNA molecules bearing a single-strand break at the EcoRI site that are resistant to further cleavage (data not shown). Production of this species was observed in experiments performed by either mixing protocol, and the fraction of input DNA converted to this species depended on the ratio of enzyme to DNA, but not on the absolute concentrations of either component. At an enzyme dimer to DNA ratio of 250:1, this species accounted for 48% of all cleaved DNA. This probably reflects low level contamination of our $Eco$ RI methylase during enzyme isolation (23), contaminating the methylase cofactor, $S$-adenosylmethionine, copurifies with $Eco$ RI methylase during enzyme isolation (23), contaminating the methylase as follows when the enzyme is used for the methylation assay, leading to an underestimate of the amount of methylase present. We have minimized this problem by performing all single turnover DNA cleavage experiments at an endonuclease to DNA ratio of 20:1 or less, conditions that result in accumulation of DNA molecules resistant to second strand cleavage at a level <10% of input DNA.
two step mechanism described above. Essentially identical results were obtained when enzyme was mixed with DNA, provided that concentrations of DNA and enzyme were chosen so that association of the endonuclease with the EcoRI site of the plasmid was not rate-limiting. The latter requirement restricted analysis under these mixing conditions to [NaCl] concentrations of 0.025–0.1 M (Table I). Over this concentration range, rate constants for first and second strand cleavage events were found to be independent of mixing protocol (Table I), ruling out a slow first order step necessary to produce a cleavage-competent endonuclease-DNA complex once the enzyme has located the EcoRI site. Furthermore, as summarized in Table I, rate constants governing the two strand cleavage steps are essentially insensitive to [NaCl] in the range of 0.025–0.2 M. Increasing ionic strength from 0.059 M to 0.23 M resulted in only a 25% decrease in the rate of first strand cleavage, whereas the rate of second strand cleavage was not significantly affected. In all cases strand cleavage rate constants are much larger than $k_{cat}$.

**Pre-steady-state Burst of Product Formation**—Comparison of pre-steady-state and steady-state results suggests that the rate-limiting step for endonuclease turnover occurs subsequent to sequence recognition and chemistry at all ionic strengths tested, predicting occurrence of a pre-steady-state burst of double-strand cleavage due to rapid formation of $E'$2 prior to the rate-limiting step. This was confirmed (Table II), although the yield was significantly less than 1 mol of double-strand breaks per mol of endonuclease dimer under all conditions tested despite that fact that DNA concentrations used were at least 5 times the $K_{on}$ value in all cases.

The less than stoichiometric burst amplitude was not due to significant levels of inactive enzyme as judged by similar experiments in which preformed endonuclease$^{[32P]}$pBR322 complexes (DNA excess) were subsequently challenged with Mg$^{2+}$ and an excess of the unlabeled form of the DNA (Table III). Under these conditions, product yield was 0.8–1.0 mol of double-strand events per mol of endonuclease dimer at ionic strengths of 0.059–0.13 M, decreasing significantly only at an ionic strength of 0.23 M. We attribute the higher product yield in this type of experiment, relative to that observed during the pre-steady-state burst, to the involvement of facilitated diffusion in the mechanism of EcoRI site location. Since pBR322 has a single recognition sequence and 4360 nonspecific sites, initial endonuclease-DNA complexes are nonspecific in nature, and these give rise to specific complexes by diffusion of the protein within the domain of the polynucleotide (19, 20). The enzyme thus kinetically partitions between nonspecific sites and the EcoRI sequence during turnover (Fig. 1A). If a subpopulation of nonspecifically bound enzyme fails to locate and cleave the EcoRI site within a time period on the order of that required for turnover, then the pre-steady-state burst will be less than unity, as we have observed. The problem of kinetic partitioning is circumvented in those experiments in which cleavage pre-formed, specific complexes is initiated by addition of Mg$^{2+}$, conditions that result in near unity product yields at low and moderate ionic strengths.

The stoichiometry of preferential cleavage of preformed complexes at ionic strengths of 0.059–0.13 M indicates that initial chemical step is fast relative to the competing dissociation reactions, i.e. $k_2 > (k_{-S} + k_{NS})$ (Fig. 1A). Under these conditions, formation of the $E$1 intermediate is therefore fast relative to conversion of specific $E$S complexes to kinetically nonproductive $E$N complexes or to dissociation of the endonuclease from the polynucleotide domain ($k_2/(k_{-S} + k_{NS}) > 10$). Since these experiments monitored double-strand cleavage events, these results also imply that second strand cleavage is tightly coupled to first strand cleavage for pBR322, as inferred previously (5, 16, 18).

The yield of preferential cleavage of preformed complexes was significantly reduced at 0.23 M ionic strength, as compared with that observed at lower salt concentrations (Table III). Because the reduced yield of double-strand cleaved product is not due to dissociation of the $E$1 intermediate at the higher ionic strength (Table I and Ref. 29), we interpret these findings in terms of partitioning of specific complexes between cleavage and departure of the endonuclease from an uncleaved site. This implies that $k_2 \approx k_{-S} + k_{NS}$ (Fig. 1A) at an ionic strength of 0.23 M.

**Effects of Processive Action on EcoRI Endonuclease Turnover**—The results described above indicate that the step that limits endonuclease turnover occurs after double-strand cleavage. We have considered three possibilities with respect to the nature of this slow step. Since the endonuclease supports processive cleavage at low to moderate ionic strengths (20), it is evident that the enzyme can leave an EcoRI site after cleavage to diffuse within the polynucleotide domain of a DNA product. Turnover may therefore be limited by the effective lifetime of this population of nonspecific complexes. A second possibility invokes slow release of the enzyme from the cleaved EcoRI site, whereas the third attributes the slow step to a rate-limiting conformational change that must occur before the endonuclease can support another round of DNA cleavage.

To address potential involvement of slow release from a cleaved site or a slow, post-cleavage conformational transition, we have exploited the previous finding that EcoRI endonuclease can act processively on DNA molecules bearing two EcoRI sites (20, 22). Because processive cleavage requires that the endonuclease leave a cleaved site, locate the second EcoRI site on the same DNA molecule, and cleave that site prior to dissociation into solution, evaluation of the $k_{cat}$ for processive events will reveal the presence of slow steps that occur between cleavage of the first and second sites. However, if turnover is limited

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3 W. Jack and P. Modrich, unpublished data.
Kinetic Mechanism of EcoRI Endonuclease

Pre-steady-state DNA cleavage as a function of NaCl concentration was performed and data analyzed as described under “Materials and Methods.” Reactions were initiated by mixing MgCl₂ with previously formed endonuclease-pBR322 complexes (ES) or by mixing pBR322 with an endonuclease MgCl₂ solution (E + S). Each value for $k_1$ or $k_2$ is the average of four determinations ± two standard deviations. ND, not determined. Values for $k_{cat}$ are from Fig. 3.

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**DISCUSSION**

Despite their significant affinity for nonspecific sequences, restriction endonucleases maintain high cleavage specificity for their canonical recognition sites and are able to locate these sequences by kinetically efficient pathways. The extensively studied EcoRI and EcoRV endonucleases address the first problem via recognition site-dependent activation of the DNA cleavage center (9, 31). Facilitated diffusion has been implicated in the pathways by which both EcoRI (19–22) and EcoRV (32, 33) endonucleases locate their recognition sites. In this type of mechanism, diffusional search occurs within the domain DNA after the initial protein-DNA collision, which favors nonspecific complex formation by a large statistical factor (34). Based on the experiments described here, we have concluded that facilitated diffusion processes dominate the steady-state kinetic behavior of the EcoRI endonuclease with natural DNA substrates. Despite the large effects of ionic strength on $K_m$ and $k_{cat}$ (Fig. 3), we have been unable to detect any significant effects of this reaction parameter on the kinetics of recognition and cleavage events that occur once the enzyme has arrived at the EcoRI sequence (Table I). We therefore attribute the in-
increases in $K_m$ and $k_{\text{cat}}$, which are associated with increased salt concentration to nonspecific endonuclease-DNA interactions that occur prior to EcoRI site location and subsequent to double-strand cleavage.

The differential effects of increases in ionic strength on $K_m$ and $k_{\text{cat}}$ can be understood in terms of the importance of nonspecific endonuclease-DNA complexes in the reaction. At low to moderate ionic strengths, positionally correlated facilitated diffusion, which is topologically equivalent to “sliding” (34), plays an important role in the mechanism by which EcoRI endonuclease locates its recognition sequence (19–21). At an ionic strength of 0.08 M, the endonuclease has been estimated to scan about 1,300 base pairs per DNA binding event by a sliding type mechanism, with the effective chain length scanned in this manner decreasing rapidly with increasing salt concentration (19). At low ionic strengths, which favor a low $K_m$, the endonuclease reaction thus approaches the diffusion limit where each protein-DNA collision event is productive with respect to DNA cleavage (see also below). The efficiency of this search process requires extensive sampling of the hexanucleotide sequence present within the substrate. The effect of salt concentration on $k_{\text{cat}}$ can be understood in similar terms. Inasmuch as the slow step in the reaction occurs subsequent to cleavage at all ionic strengths tested and since analysis of processive turnover of the enzyme has ruled out a slow conformational change or slow release from the cleaved site as rate determining, we ascribe the slow step to the lifetime of the population of non-specific complexes that result from diffusion of the protein within the domain of the DNA product prior to its dissociation into solution. This implies that DNA products are also subject to extensive sequence sampling at low ionic strength prior to rate-limiting dissociation, resulting in a low $k_{\text{cat}}$. Conversely, high ionic strength, which favors reduced nonspecific affinity and less efficient positionally correlated search (19, 20), results in elevation of both $k_{\text{cat}}$ and $K_m$. Previous work has shown that the endonuclease supports processive cleavage of EcoRI sites separated by 50–300 base pairs, with the efficiency of processive action decreasing from about 80% at an ionic strength of 0.06 M to about 30% at 0.13 M (20), and we have confirmed these findings. However, processive action has not been detected at ionic strengths of 0.18 M or higher (Table IV and Ref. 20). Nevertheless, the results described here indicate that product release is rate-limiting for turnover at ionic strengths of 0.05–0.18 M, and largely rate determining at 0.23 M (Table I). Despite the reduction in processive action observed with increases in salt concentration, the latter findings indicate that the enzyme rarely dissociates from the cleaved EcoRI site directly into solution, but rather diffuses within the domain of a polynucleotide product prior to release, even at high ionic strengths ($k_{\text{PN}} \gg k_m$, Fig. 1A). The reduced processivity at the higher salt concentrations can be explained in several ways. In order for processive action to occur, the DNA segment spanning the EcoRI sites in question must be subject to efficient sequence sampling, e.g. by positionally correlated diffusion, and this must be coupled with productive recognition of an EcoRI sequence upon encounter by the enzyme. A reduction in the efficiency of EcoRI site recognition at the higher ionic strengths may account for the failure to detect processive action of the enzyme under these conditions. A second explanation for reduced processivity under conditions where the endonuclease remains associated with the DNA product is based on the potential involvement of a distinct mode of sequence sampling at high ionic strength. In contrast to positionally correlated microscopic dissociation-reassociation (sliding), the intersegment transfer (macroscopic dissociation-reassociation) mode of facilitated diffusion invokes ligand transfer between distal DNA sites that happen to be in proximity due to the flexibility of large DNA chains (34). Thus, reduction in the DNA segment size that is subject to positionally correlated search coupled with intersegment transfer of the endonuclease within the domain of a DNA product could account for the reduction in processive behavior observed at high ionic strength under conditions where the endonuclease nevertheless remains associated with a cleavage product.

Equations relating the steady-state kinetic parameters $K_m$ and $k_{\text{cat}}$ to the rate constants shown in the reduced mechanism of Fig. 1B can be calculated as shown by Equations 2 and 3:

$$K_m = \frac{(k_3 + k_{\text{cat}}) k_3 k_{\text{cat}}}{k_1 (k_3 + k_4 k_{\text{cat}} + k_4)} \quad (\text{Eq. 2})$$

$$K_m = \frac{k_3 k_{\text{cat}}}{k_3 + k_4} \quad (\text{Eq. 3})$$

The ratio $k_{\text{cat}}/K_m$ is given by Equation 4:

$$\frac{k_{\text{cat}}}{K_m} = \frac{k_3 k_4}{k_3 + k_4} \quad (\text{Eq. 4})$$

We have used Equation 3 to compare measured values of $k_{\text{cat}}$ to the first order rate constant governing product release, $k_1$, at several ionic strengths using experimental values for $k_{\text{cat}}$, $k_2$, and $k_3$ (Fig. 3 and Table I). Measured values of $k_{\text{cat}}$ are 1.0–1.1 times $k_4$ at ionic strengths of 0.18 M or less, and 1.27 times $k_4$ at an ionic strength of 0.23 M, consistent with the conclusion above that dissociation of the endonuclease from the DNA product is rate-limiting for turnover. These values for $k_4$, experimental values for $k_2$ and $k_3$ (Table I), and published values for $k_1$ and $k_{-1}$ (7, 19, 28) allow calculation of $K_m$ according to Equation 2. For these calculations, $k_1$ and $k_{-1}$ were corrected for the effects of 5 mM MgCl$_2$ ($k_{-1}/10$ and $k_{-1} \times 3$) based on the effects of the divalent cation on the kinetics of association and dissociation of a mutant form of the endonuclease that retains effects of the divalent cation on the kinetics of association and dissociation (15). Table V shows that these calculated values (designated $K_m^{*}$) are within a factor of 4 of the measured $K_m$ values over the ionic strength range of 0.059 to 0.18 M. Table V also compares $k_{\text{cat}}/K_m$ values calculated in a similar manner according to Equation 4. These values ($k_{\text{cat}}/K_m$) are within factors of 2–5 of measured values. The close agreement of calculated and experimental values for $K_m$ and $k_{\text{cat}}/K_m$ strongly suggests that the kinetic scheme depicted in Fig. 1B is a valid description of the EcoRI endonuclease kinetic mechanism.

Under conditions where $k_2 \gg k_{-1}$ (Fig. 1B), Equation 4 reduces to Equation 5:

$$\frac{k_{\text{cat}}}{K_m} = k_1 \quad (\text{Eq. 5})$$

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<td>1.1 × 10$^{-9}$</td>
<td>1.4 × 10$^{-9}$</td>
<td>6.3 × 10$^{-9}$</td>
</tr>
<tr>
<td>0.20</td>
<td>0.23</td>
<td>1.1 × 10$^{-7}$</td>
<td>ND</td>
<td>2.0 × 10$^{-9}$</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Table V Measured and calculated parameters governing steady-state cleavage by EcoRI endonuclease*
This relationship describes a diffusion-controlled reaction, in which each encounter between an enzyme and its substrate leads to reaction. At ionic strengths of 0.13 M or less, $k_2 > k_1$ and $k_{cat}/K_m$ approximates $k_1$ when values of $k_1$ and $k_{-1}$ are corrected for effects of MgCl$_2$ (see above). This suggests that EcoRI endonuclease cleavage of pBR322 is diffusion-controlled or nearly so under these conditions. The large increase in EcoRI endonuclease cleavage of pBR322 is diffusion-controlled DNA.

The kinetic mechanism of EcoRI endonuclease bears similarities to that of EcoRV endonuclease, another well studied type II restriction enzyme, but also some interesting differences. As mentioned above, facilitated diffusion has been implicated in the kinetic path by which both enzymes locate their recognition site (19–22, 32, 33), both catalyze sequential strand cleavage reactions (5, 35), and product release appears to limit the rate of turnover for both enzymes (this work and Refs. 5 and 35). However, it has been reported that EcoRV endonuclease requires divalent metal ions such as Ca$^{2+}$ or Mg$^{2+}$ for site-specific DNA binding and only binds nonspecifically in their absence (36–38), although weak specific binding has been reported under some reaction conditions (39). EcoRI endonuclease clearly requires Mg$^{2+}$ only for steps occurring after site-specific binding. Interestingly, whereas EcoRI can catalyze processive DNA cleavage, verifying that it can be transferred between recognition sites after catalyzing a round of DNA cleavage without dissociating from the DNA product, it appears that EcoRV endonuclease does not catalyze processive cleavage of two recognition sites located on the same DNA molecule (33). This suggests that EcoRV either dissociates from a cleaved site into solution without diffusing onto neighboring nonspecific DNA sequences or that it is unable to support a subsequent round of DNA cleavage without first dissociating from the DNA chain, perhaps due to a requirement for a conformational transition that cannot occur while the enzyme remains bound to DNA.

REFERENCES