Kinetic Characterization of Linear Diffusion of the Restriction Endonuclease *Eco*RV on DNA†

Albert Jeltsch and Alfred Pingoud*

Institut für Biochemie, FB Biologie, Justus-Liebig Universität, Heinrich-Buff-Ring 58, 35392 Giessen, Germany

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ABSTRACT: We have examined the kinetic parameters of linear diffusion of *Eco*RV on DNA. The data were analyzed by Monte Carlo simulations in which the efficiency of recognition of *Eco*RV sites during linear diffusion, the efficiency of linear diffusion, and the behavior of enzymes at the ends of linear DNA is explicitly treated. The analysis of the dependence of linear diffusion on the concentrations of NaCl and $MgCl_2$ shows that linear diffusion is maximal at 50 mM NaCl under all concentrations of $MgCl_2$ tested and increases with increasing concentrations of Mg^{2+} up to 10 mM, the highest concentration used in the test. Under these conditions, *Eco*RV scans 2×10^6 bp during one binding event with a velocity of about 1.7×10^6 bp s^{-1}. The enzyme tends to overlook cleavage sites at 1 mM but not at 10 mM $MgCl_2$. This result confirms the thermodynamic finding that *Eco*RV does not bind very specifically to DNA in the absence of Mg^{2+}. It demonstrates that there is a Mg^{2+}-dependent continuous transition between a nonspecific and a specific binding mode of *Eco*RV to DNA. By comparing cleavage rates of linear DNA whose ends are free or blocked, we have shown that *Eco*RV has a very low probability to fall off at the ends of linear DNA. The enzyme rather is "reflected" and continues linear diffusion. *Eco*RV does not cleave oligonucleotides containing two *Eco*RV sites processively. Consequently, dissociation of the enzyme from the cleavage products is not preceded by a transfer to nonspecific DNA, and linear diffusion is not involved in product dissociation in *Eco*RV.

The *Eco*RV restriction endonuclease highly specifically cleaves DNA at GAT↓ATC sites. It is a component of a type II restriction/modification system (for reviews, see refs *1* and *2*). Type II restriction endonucleases (for reviews, see refs *3*−*5*) defend bacteria against bacteriophages by cleaving the phage DNA immediately after infection. The bacterial DNA is protected from cleavage by a methylation within the recognition site introduced by a corresponding type II methyltransferase (for reviews, see refs *6* and *7*). To fulfill its biological function, the restriction enzyme must find its recognition sites on the phage DNA, before the phage DNA becomes methylated and before it can start its deleterious action in the cell. However, fast target site location is not easy to achieve because the diffusion rate of the enzyme is limited (*8*−*10*). Moreover, the restriction enzyme has to find its specific sites in a large background of nontarget DNA. *Eco*RV, like many other restriction endonucleases, makes use of a highly developed strategy of target site location. First, it binds nonspecifically to DNA and then it scans the DNA for specific sites (*11, 12*). This search is a thermally driven one-dimensional diffusion process, best described as a random walk. Due to the reduction in dimensionality, it requires much less time than a three-dimensional search in solution would do (*13*−*15*; for reviews, see refs *16* and *17*).

Facilitated diffusion of proteins on DNA is a general phenomenon (for review, see ref *18*) that in addition to restriction endonucleases [*Bam*HI (*19, 20*), *Bss*HII (*21*), *Eco*RI (*19, 22*−*24*), *Eco*RV (*11, 12*), *Hin*dIII (*19*)] has been observed with many other proteins such as nucleases [UV-endonuclease (*25*), T4 endonuclease V (*26*−*28*), BAL31 nuclease (*29*)], RNA polymerase (*30, 31*), DNA methyltransferases [M·*Bam*HI (*20*), M·*Eco*RI (*32, 33*)], repressors [lac repressor (*34*−*37*), λ cro protein (*38*)], and transcription factors [TATA binding protein (*39*), steroid receptor (*37*)]. The ability of proteins to make use of facilitated diffusion has been shown to be of fundamental importance for the in vivo functions of T4 endonuclease V (*40, 41*) and *Eco*RV (*12*).

A detailed kinetic analysis of facilitated diffusion of restriction endonucleases has so far been only carried out with *Eco*RI. It revealed that (i) the protein scans about 1000 bp by linear diffusion in one DNA binding event (*19*), (ii) the number of bp scanned is dramatically reduced at 10 mM $MgCl_2$ as compared to 1 mM $MgCl_2$ (*19*), (iii) the enzyme follows the helical pitch of the DNA (*24*), (iv) it does not overlook any recognition site on its route (*24*), (v) it pauses at sites that resemble its recognition site (*24*), and (vi) linear diffusion is involved in product dissociation (*22*).

Here, we investigate the contribution of linear diffusion to the mechanism of DNA cleavage by *Eco*RV. It complements previous studies carried out with *Eco*RI (*19, 24*) and *Eco*RV (*12*) by addressing questions that so far have not been investigated, viz. (i) whether the enzyme falls off at the ends of linear DNA, (ii) to what extent the range of linear

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* Corresponding author phone +49 641 99 35400, fax: +49 641 99 35409.

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diffusion of *Eco*RV depends on the ionic milieu, (iii) how DNA recognition is achieved during the linear diffusion process, and (iv) whether product release after DNA cleavage is influenced by linear diffusion.

EXPERIMENTAL PROCEDURES

Proteins and Oligonucleotides. *Eco*RV was purified essentially as described (*42*), except that an additional chromatography step over phosphocellulose was carried out. HPLC-purified oligonucleotides were obtained from Interactiva (Ulm, Germany).

Competitive Cleavage Assay of Two Substrates. To determine the relative rates of cleavage of two substrates, a competitive cleavage assay was employed. This assay has been described in detail (*43*) and already applied for *Eco*RI (*24*) and *Eco*RV (*12*). Briefly, the cleavage rates of a PCR product and a 26mer oligonucleotide each containing one *Eco*RV site are determined in competition. Both substrates carry a different label on one end allowing us to detect the disappearance of both substrates in one reaction mixture independently and very accurately. Oligo26 [d(TGGCGCC**GATATC**GCCGACATCAAAG)] is used as the upper primer to amplify different PCR products from the pAT153 template. Appropriate lower primers serve to amplify fragments 158, 498, 958, 1488, and 2092 bp in length (Figure 1) (*12*). A 26mer oligonucleotide substrate was generated by annealing oligo26 with a complementary oligonucleotide. Therefore, in all of these substrates the *Eco*RV site is embedded in an identical sequence context of 26 bp, but flanked by an arm comprising between 0 and 2066 bp. Thus, differences in the cleavage rates between the 26mer and the different PCR products can only be due to the different length of the two substrates and are correlated with linear diffusion of *Eco*RV along the DNA. In the cleavage reactions, 20 nM 26mer and 10 nM PCR product were cleaved with 8 nM *Eco*RV in 20 mM Tris-HCl, pH 7.5, 50 μg/mL BSA at 21 °C. $MgCl_2$ and NaCl were added in the concentrations as indicated. If not otherwise stated, the concentration of NaCl was 50 mM.

Cleavage Reactions Using Substrates Containing Two Adjacent EcoRV Sites. To find out if *Eco*RV tends to overlook recognition sites during linear diffusion, the cleavage rates of two substrates each containing two *Eco*RV sites were measured. A 32mer oligonucleotide [oligo-2sites: d(GCGCC**GATATCGATATC**ATCACCGATGGGGAA)] was labeled on its 5′- or 3′-end using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (MBI Fermentas) or [α-^{32}P]ddATP (Amersham) and terminal deoxynucleotidyl transferase (MBI Fermentas). Oligo-2sites was hybridized with a complementary oligonucleotide to generate the 32 bp substrate 2sitesI. A 496mer substrate (2sitesII) was generated by PCR from pAT153 using 5′-labeled oligo-2sites and the corresponding lower primer (Figure 1). The sample of 20 nM substrate 2sitesI or 2sitesII was incubated with 10 nM *Eco*RV in cleavage buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 μg/mL BSA) containing 1 mM or 10 mM $MgCl_2$ at 21 °C. After appropriate time intervals, aliquots were withdrawn from the reaction mixture, spotted onto DEAE thin-layer plates (Macherey & Nagel, Düren) and subjected to homochromatography (*44*). Homochromatograms were quantitatively analyzed using an instant imager (Canberra Packard). The initial DNA cleavage rates at the two sites were determined from the initial rates of formation of the corresponding cleavage products.

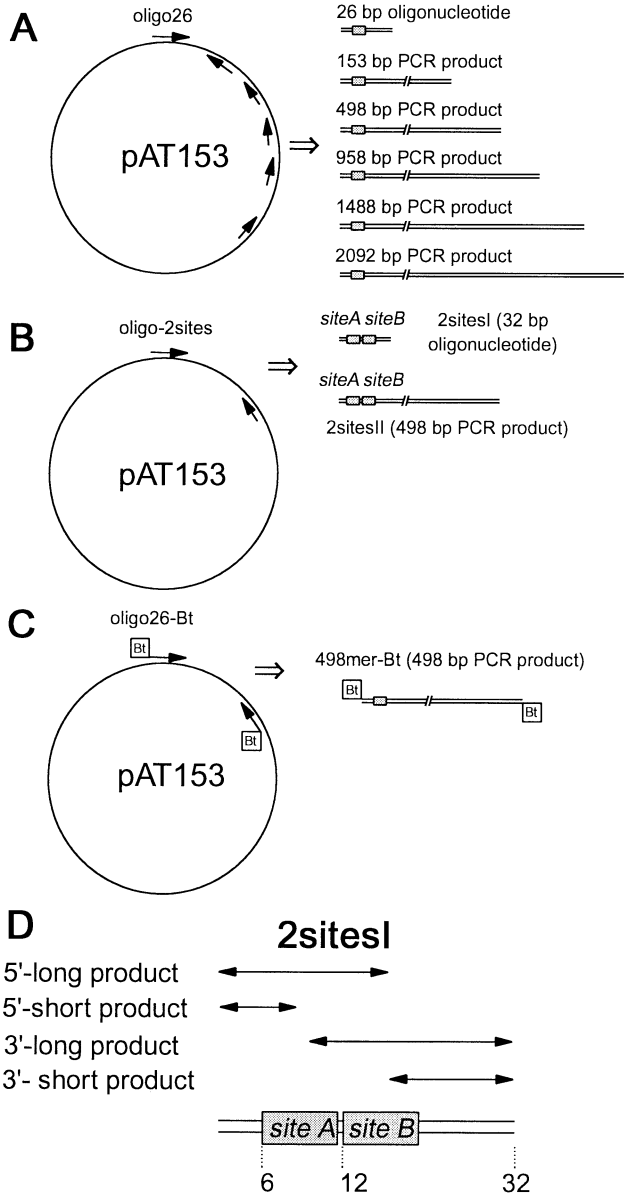

FIGURE 1: Compilation of substrates used in this study (DNA fragments are not drawn to scale). (A) Five substrates each containing one *Eco*RV site were generated by PCR using pAT153 as template and oligo26 as upper primer, which was also used to generate a 26mer oligonucleotide substrate. (B) One 498mer substrate (2sitesII) containing two *Eco*RV sites was generated by PCR using oligo-2sites as upper primer. Substrate 2sitesI was produced by annealing oligo-2sites and a complementary oligonucleotide. (C) One 498mer substrate (498mer-Bt) was generated by PCR using biotinylated PCR primers. (D) Fragments resulting from *Eco*RV cleavage of substrate 2sitesI. Cleavage at site A produces a 5′-short product and a 3′-long product; cleavage at site B results in a 3′-short product and a 5′-long product. Depending on whether the 5′ or the 3′ end of the upper strand of the substrate is labeled, only one of the long products and only one of the short products is detectable.

Cleavage Reactions Using Linear DNA Substrates with Blocked Ends. To obtain DNA substrates with blocked ends, biotinylated oligonucleotides were used to obtain by PCR a 498mer substrate that carries one biotin on each end (498mer-Bt) (Figure 1). The degree of biotinylation of each oligo-

nucleotide was shown to be better than 95% by denaturing polyacrylamide gel electrophoresis of the oligonucleotides (data not shown). Cleavage experiments using 10 nM radioactively labeled DNA and 10 nM *Eco*RV were performed in cleavage buffer containing 10 mM $MgCl_2$ in the presence (40 nM) or absence of avidin (Sigma). As one avidin molecule is able to bind to four biotin residues, this corresponds to an 8:1 molar excess of avidin over biotin. Given the very high binding constant of biotin to avidin, each biotin will be bound to an avidin. To allow complete binding of avidin to the biotin residues, the reaction mixtures containing avidin were incubated for 30 min prior to the addition of *Eco*RV. The cleavage reactions were analyzed as described in the previous paragraph.

Nonspecific Binding of EcoRV to DNA. DNA binding was analyzed by nitrocellulose filter binding experiments carried out in a dot blot apparatus (Bio-Rad) similarly as described by Wong and Lohman (*45*). A 20mer substrate not containing an *Eco*RV site (dGATCGACGAGCTCGTCGATC) was end labeled using [γ-^{32}P]ATP (Amersham) and polynucleotide kinase (MBI, Fermentas). The 5 nM labeled DNA was incubated with different amounts of *Eco*RV (0−600 nM) in 50 μL of 50 mM Tris-HCl, pH 7.5, for 30 min at ambient temperature. NaCl and $MgCl_2$ were added as indicated in the text. The nitrocellulose filter membrane (Macherey & Nagel, Düren) was prerinsed with incubation buffer for 30 min. After the sample was transferred into the dot blot chamber, the slots were washed twice with 100 μL of incubation buffer. The samples were transferred into the wells of the dot blot apparatus using a multiple pipet, immediately suck through the nitrocellulose filter membrane, and washed several times with 100 μL of incubation buffer. The radioactivity of the spots was analyzed using an Instant Imager (Canberra Packard).

Theoretical Analysis of Linear Diffusion. In all analyses, we have assumed that *Eco*RV covers 10 bp on the DNA as seen in the X-ray structure (*46*). This means that a DNA with L[1] bp provides $L - 10$ nonspecific binding sites.[2] Throughout this paper, we have assumed that all nonspecific sites are of equal affinity for *Eco*RV.

Assuming that association can occur with a rate k_{+1} at any site n, that the specific binding site is located at position s, and that each encounter with the specific site leads to cleavage, the overall association rate to the specific site (k_{on}) only depends on the probability P_{dif} that the enzyme diffuses one step along the DNA rather than dissociates from the DNA. P_{dif} is equal to k_{dif}/k_{-1}, the ratio of the rate constants for linear diffusion (k_{dif}) and dissociation (k_{-1}). Then, the contribution of linear diffusion to the rate of association between *Eco*RV and its recognition site on a DNA with a length of L bp can be described by (*19*):

$$k_{on}/k_{+1} = \sum_{n=1}^{L} \exp[-(n - s)^2/P_{dif}] \quad (1)$$

with k_{on} as the overall association rate constant to the specific site, k_{+1} as the association rate constant of *Eco*RV to nonspecific sites, L as the effective length of the DNA, s as the position of the specific site, and P_{dif} as the probability to diffuse one step along the DNA rather than to dissociate from the DNA. P_{dif} corresponds to the average number of linear diffusion steps of the enzyme during one binding event. The average number of base pairs scanned by *Eco*RV in one binding event is given by the square root of P_{dif}.

This equation has two drawbacks: it describes a movement on an infinite linear lattice and it presumes that *Eco*RV always recognizes and cleaves the recognition site after associating to it. To overcome these limitations, we have analyzed linear diffusion by Monte Carlo simulations, using a program that simulates a one-dimensional random walk of an enzyme on a DNA−substrate providing L binding sites. After association to the DNA, the protein molecule starts a stepwise random walk, the reach of which is defined by the probability to carry out one linear diffusion step or to dissociate (P_{dif}, $P_{dif} = k_{dif}/k_{-1}$). If ends are encountered, the molecules are reflected or dissociate as defined by the probability of reflection P_{ref}. After an association to the cleavage site, the enzyme has a certain probability to recognize the site (P_{rec}). If the site is recognized, the association is productive; otherwise the random walk is continued until the enzyme finally recognizes a cleavage site or falls off from the DNA. Five associations to each position of the substrate were simulated and the results averaged. The total number of productive associations to each substrate defines the rate enhancement of DNA cleavage due to linear diffusion. The theoretical association rates to all different substrates were normalized by multiplication with an appropriate factor to make them comparable to the experimental cleavage rates. Different Monte Carlo simulations were carried out for all substrates used to determine the values of P_{dif}, P_{ref}, and P_{rec} that match the experimental results.

RESULTS

In this study, we have analyzed (i) the probability of *Eco*RV to fall off at the ends of linear DNA, (ii) the NaCl and $MgCl_2$ dependence of linear diffusion, (iii) the efficiency of DNA recognition by *Eco*RV at different concentrations of $MgCl_2$, and (iv) the mechanism of product dissociation from *Eco*RV.

"Reflection" of EcoRV at the Ends of Linear DNA. It is not known whether a restriction enzyme when diffusing along the DNA falls off at the ends of linear DNA or is reflected. We, so far, have favored an "end reflection" model, but without any experimental evidence for it (*12*, *19*, *24*). However, it has been reported recently that the *Eco*RI DNA methyltransferase, which is also capable of linear diffusion along DNA, prefers to dissociate from a linear DNA when it approaches the end (*33*). This raises the interest in finding out if *Eco*RV behaves similar as M·*Eco*RI. The experimental test system to find out if *Eco*RV dissociates or is reflected at the ends of DNA fragments is very straightforward. Cleavage experiments using the 498mer-Bt substrate which carries one biotin residue at each end were carried out in

[1] k_{on}, overall association rate constant to the specific site; k_{+1}, association rate constant of *Eco*RV to nonspecific sites; k_{-1}, dissociation rate constant of *Eco*RV from nonspecific DNA; k_{dif}, rate constant for linear diffusion; L, effective length of the DNA; P_{dif}, probability of the enzyme to diffuse one step along the DNA rather than to dissociate from the DNA; P_{rec}, probability of the enzyme to recognize a cleavage site after associating to it; P_{ref}, probability of the enzyme to be reflected at the ends of linear DNA; P_t, probability of the enzyme to be transferred to nonspecific DNA after DNA cleavage.

[2] For example, a 50mer with a GATATC site whose G is located at bp 10 will be treated as a 40mer that has a recognition site at position 8.

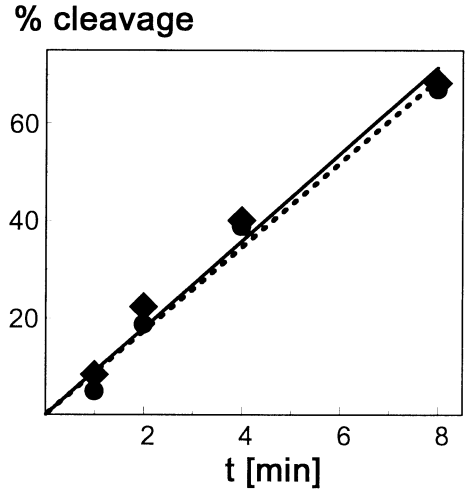

FIGURE 2: DNA cleavage rates of the biotinylated substrate in the absence (●, ···) and presence (◆, —) of avidin. The samples of 10 nM 498mer-Bt and 10 nM *Eco*RV were incubated in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 50 μg/mL BSA, and their cleavage was analyzed by homochromatography. Note that the presence of avidin does not influence the DNA cleavage rate of *Eco*RV.

Table 1: Linear Diffusion of Wild-Type *Eco*RV Restriction Endonuclease under Various Buffer Conditions[a]

v_{958mer}/v_{26mer}		*c*(NaCl) (mM)			
		0	20	50	100
c($MgCl_2$) (mM)	1	1.2	1.7	5.6	2.5
	2	1.8	2.9	6.5	2.9
	5	3.6	4.7	6.8	4.5
	10	4.8	5.6	7.8	4.6

[a] The relative rates of cleavage of the 958_{mer} and 26_{mer} are given (v_{958mer}/v_{26mer}). All experiments were carried out at least in duplicate. The values given are accurate within ±0.5.

the presence as well as in the absence of avidin. As avidin has a molecular mass of 67 kDa, *Eco*RV cannot fall off at the end of a linear DNA that is blocked by avidin. In contrast, biotin has a molecular mass of <0.5 kDa and is unlikely to disturb dissociation at the ends of linear DNA, if it occurred. In agreement with this assumption, a 498mer DNA whose ends are not biotinylated is cleaved with the same rate as the biotinylated 498mer substrate (data not shown). In addition, the mean rates of DNA cleavage in the presence of avidin (v_{+av}) and in the absence of avidin (v_{-av}) are almost identical ($v_{+av}/v_{-av} = 1.02 \pm 0.015$) (experiments were carried out in triplicate, see Figure 2 for an example). The very small rate enhancement observed in the presence of avidin, if considered significant, can be used to estimate the upper limit of the probability of *Eco*RV to leave the DNA upon approaching an end to be 2%. This result indicates that dissociation at the ends of linear DNA does not occur during most catalytic cycles of *Eco*RV.

Dependence of the Efficiency of Linear Diffusion on the Concentrations of $MgCl_2$ and NaCl. As linear diffusion is dependent on nonspecific binding of *Eco*RV to DNA, one would expect a strong dependence on the concentrations of mono- and divalent cations. We have determined the cleavage rate of a 958mer DNA fragment in competition with a 26mer oligodeoxynucleotide at different concentrations of Mg^{2+} and Na^+ (Table 1). The results can be summarized as follows: (a) under all NaCl concentrations investigated (0−100 mM), *increasing* the concentration of $MgCl_2$ results in an *increased* efficiency of linear diffusion, and (b) under all concentrations of $MgCl_2$ linear diffusion was maximal at 50 mM NaCl.

Table 2: Nonspecific DNA Binding Constants of *Eco*RV[a]

conditions	$K_{Ass,ns}$ (M^{-1})
1 mM $MgCl_2$, 20 mM NaCl	3.0×10^6
1 mM $MgCl_2$, 50 mM NaCl	4.4×10^6
1 mM $MgCl_2$, 100 mM NaCl	2.0×10^5
10 mM $MgCl_2$, 20 mM NaCl	8.1×10^5

[a] The $K_{Ass,ns}$ values refer to the binding of *Eco*RV to nonspecific 20_{mer}. 5 nM DNA was incubated with *Eco*RV in 50 mM Tris-HCl, pH 7.5, and NaCl; $MgCl_2$ was added as indicated. All experiments were carried out in duplicate; the values given are valid ±20%.

The k_{958mer}/k_{26mer} values are correlated to the probability P_{dif} of the enzyme to diffuse over one base pair instead of dissociating from the DNA, which is given by the ratio of the rate constants for both processes: $P_{dif} = k_{dif}/k_{-1}$. To get an estimate of the value for k_{-1}, we have measured the nonspecific DNA binding constants of *Eco*RV under some of these conditions (Table 2). The results show that the binding constant is reduced at higher concentrations of $MgCl_2$ and NaCl (>50 mM) but also at low concentrations of NaCl. Similar *c*(NaCl) dependencies of K_{Ass} have been observed with other DNA binding proteins (*35*, *47*).

The dependence of the efficiency of linear diffusion on NaCl is a generalization of results reported earlier (for 1 mM $MgCl_2$) (*12*). By comparing Tables 1 and 2, it becomes clear that the c(NaCl) dependence of linear diffusion has the same tendency as that of nonspecific binding, suggesting that the decrease in k_{dif}/k_{-1} observed at concentrations of NaCl that differ from 50 mM is mainly due to an increase in k_{-1}. In contrast, the dependence of linear diffusion on the concentration of Mg^{2+} was a completely unexpected result, because Mg^{2+} reduces the nonspecific DNA binding affinity of *Eco*RV (*48*; Table 2). Thus, one would have expected, that with increasing concentrations of Mg^{2+}, the dwell time of the protein on the DNA would decrease and, consequently, the efficiency of linear diffusion should be reduced, as found with *Eco*RI (*19*). However, in the case of *Eco*RV a reduction of linear diffusion by increasing *c*(Mg^{2+}) is not observed at concentrations below 10 mM, although there is no doubt that it must occur at higher concentrations of $MgCl_2$.

To investigate in more detail the unexpected finding that the efficiency of linear diffusion is enhanced at higher concentrations of Mg^{2+}, we have determined the rates of cleavage of five DNA fragments of different length in competition with a 26mer oligonucleotide in the presence of 1 mM as well as 10 mM $MgCl_2$. The results (Figure 3) show that longer substrates are cleaved faster than shorter ones and that this effect is more pronounced at 10 mM than at 1 mM $MgCl_2$. On the basis of the results shown in Figure 3, the maximal range of linear diffusion can be estimated to be ≈1000 bp at 1 and >2000 bp at 10 mM $MgCl_2$.

Efficiency of Recognition at Different Concentration of $MgCl_2$. The results presented so far demonstrate that there is a principal difference between *Eco*RV and *Eco*RI regarding the Mg^{2+} dependence of linear diffusion, because the ability of *Eco*RI for linear diffusion on DNA was reduced in the presence of 10 mM $MgCl_2$ (*19*), whereas it is increased in the case of *Eco*RV. It is tempting to speculate that this

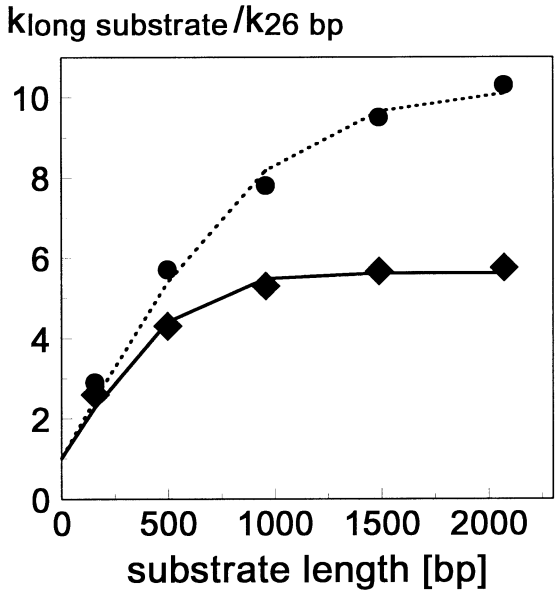

FIGURE 3: Length dependence of DNA cleavage rates by *Eco*RV in the presence of 1 (◆, —) and 10 mM $MgCl_2$ (●, ···). The relative rates of cleavage of the PCR fragments ($k_{long\ substrate}/k_{26\ bp}$) were determined in competition with the 26mer oligonucleotide in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 50 μg/mL BSA. The solid and dotted lines are theoretical values determined by Monte Carlo simulations.

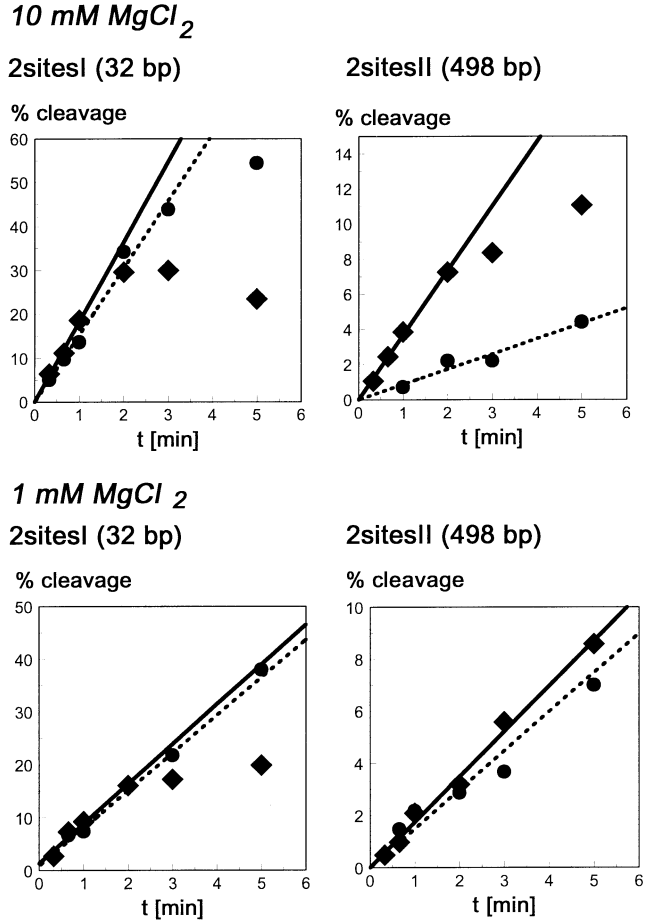

FIGURE 4: Rates of DNA cleavage of substrates containing two *Eco*RV sites: 2sitesI (left column) and 2sitesII (right column). The DNA is labeled only on the 5′ end of the upper strand. In both cases two products can be observed, one long product (◆, —), resulting from cleavage at site B, and one short product (●, ···), resulting from cleavage at site A. As the long product still contains one *Eco*RV site, its concentration decreases after longer incubation times. Cleavage reactions are carried out in buffer containing 10 mM (upper set of panels) or 1 mM $MgCl_2$ (lower set of panels). In each case, 20 nM substrate and 10 nM *Eco*RV were incubated in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 50 μg/mL BSA, and their cleavage was analyzed by homochromatography. All experiments were carried out twice. The relative rates of cleavage at sites A and B were determined to be $v_B/v_A = 1.16 \pm 0.06$ (2sitesI, 1 mM $MgCl_2$), 1.41 ± 0.1 (2sitesI, 10 mM $MgCl_2$), 1.20 ± 0.05 (2sitesII, 1 mM $MgCl_2$), and 4.9 ± 0.1 (2sitesII, 10 mM $MgCl_2$).

difference is caused by the different functions of Mg^{2+} for the recognition process in both systems. Whereas *Eco*RI binds highly specifically to DNA in the absence of Mg^{2+} (*49*−*52*), *Eco*RV does not (*11*, *48*, *53*), but requires Mg^{2+} (*48*, *54*) or Ca^{2+} (*55*) for specific binding. This means that *Eco*RI needs Mg^{2+} mainly for catalysis, whereas in *Eco*RV Mg^{2+} also contributes to DNA recognition. For *Eco*RI (*24*) and *Bss*HII (*21*), it was shown that these enzymes do not "overlook" any target site during the diffusional walk. Thus, these enzymes have a very high efficiency of recognition, which means that the probability to recognize (and cleave) a specific site after binding to it is high. In contrast, for *Eco*RV it is plausible to assume that under low concentrations of Mg^{2+} the diffusional search for cleavage sites is impaired by the inability of the enzyme to recognize the target sequence. To test this hypothesis, we have analyzed the DNA cleavage rates of two DNA substrates both containing two *Eco*RV sites immediately adjacent to each other. One substrate is 32 bp in length (2sitesI), the other is 496 bp (2sitesII) (cf. Figure 1). It should be noted that the *Eco*RV sites are in an identical sequence context in both substrates. The only difference between the substrates 2sitesI and 2sitesII is that site B is flanked by a long stretch of DNA in substrate 2sitesII but not in 2sitesI. If *Eco*RV efficiently recognized specific sites, one would expect that site B is cleaved faster than site A in substrate 2sitesII but not in 2sitesI. Our results (Figure 4) demonstrate that *Eco*RV efficiently recognizes its cleavage site at 10 mM $MgCl_2$ because under these conditions site B is cleaved 4.9 times faster than site A in substrate 2sitesII but only 1.41 times faster in 2sitesI. In contrast, at 1 mM $MgCl_2$ the enzyme does not efficiently recognize its cleavage site, because under these conditions the ratios of cleavage rates at sites A and B are almost identical for the substrates 2sitesI (1.16) and 2sitesII (1.20). We conclude that linear diffusion speeds up the overall cleavage process much more at high than at low concentrations of Mg^{2+}, because of the failure of the enzyme to efficiently recognize its specific sites at low concentrations of Mg^{2+}.

Determination of the Microscopic Probabilities for Linear Diffusion, Site Recognition, and End Reflection by Monte Carlo Simulations. The data given in Figures 2−4 were analyzed by Monte Carlo simulations to find out the intrinsic probabilities for linear diffusion (P_{dif}), end reflection (P_{ref}), and site recognition (P_{rec}). To this end, the data given in Figure 3 were analyzed by simulation of linear diffusion on five different substrates comprising 158, 498, 958, 1488, and 2092 bp to determine the number of productive associations to each substrate, which is correlated to the length dependence of the DNA cleavage rate of *Eco*RV. The results presented in Figure 4 were analyzed by simulating linear diffusion on a 496mer that contains two cleavage sites at positions 6 and 12. The number of associations to both sites were counted, and the relation $k_{ass,A}/k_{ass,B}$ was calculated. In

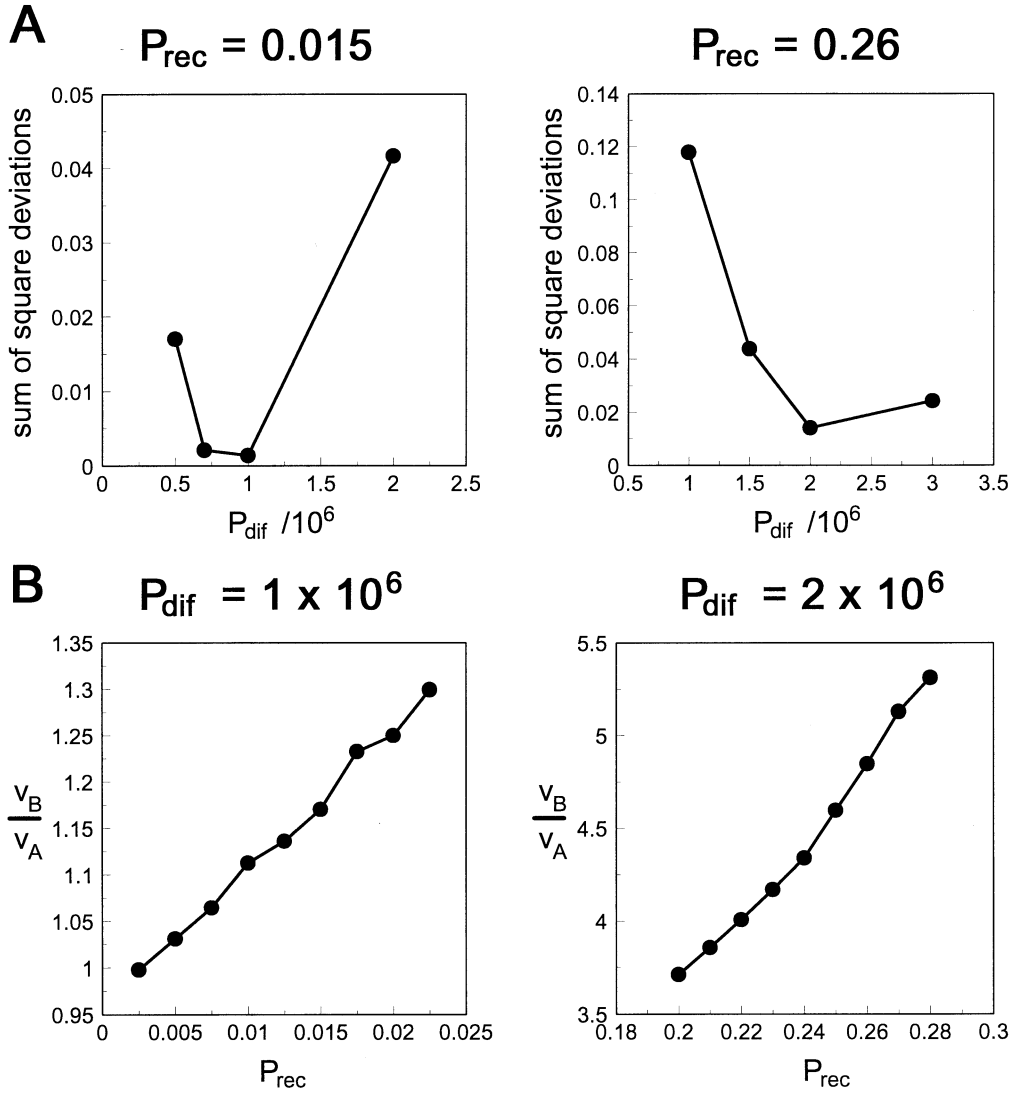

FIGURE 5: Results of the Monte Carlo simulations of linear diffusion. (A) Analysis of the length dependence of the DNA cleavage rate of *Eco*RV. The data given in Figure 3 were compared with values determined by Monte Carlo simulations using different P_{dif} values and a P_{rec} of 0.015 or 0.26, respectively, valid at 1 or 10 mM $MgCl_2$. In the figure, the sums of the squares of the deviations between calculated and experimental values are plotted against the P_{dif} values used in the simulation. P_{dif} values of 1×10^6 and 2×10^6 best match the experimental results obtained in the presence of 1 and 10 mM $MgCl_2$. (B) Analyses of the probability of *Eco*RV to recognize a cleavage site after associating to it on a substrate containing two *Eco*RV sites (2sitesII). Using P_{dif} values of 1×10^6 and 2×10^6, different values of P_{rec} were used to determine by simulation theoretical ratios of cleavage rates at sites B and A. According to our simulation, the experimentally determined v_B/v_A values of 1.2 and 4.9 at 1 and 10 mM $MgCl_2$ (Figure 4) correspond to P_{rec} values of 0.015 and 0.26.

these simulations all three parameters (P_{dif}, P_{ref}, and P_{rec}), in principle, are interdependent, so we used an iterative fitting strategy in which always only one parameter was changed. We have shown above that end dissociation must be a rare event so that we set $P_{ref} = 1$ for the first series of simulations. Then, we have used P_{dif} values determined by eq 1 ($P_{dif} = 1.2 \times 10^6$ in 10 and 3×10^5 in 1 mM $MgCl_2$) to calculate P_{rec} values at 10 and 1 mM $MgCl_2$ that best reproduce the experimentally determined ratios of cleavage rates at sites B and A in substrate 2sitesII (Figure 4). Subsequently, the resulting P_{rec} values were used to find by simulation better matching P_{dif} values using the data on the length dependence of the DNA cleavage rate (Figure 3) and so on. Some results of the Monte Carlo analyses of the data are shown in Figure 5. After three cycles, we obtained two pairs of P_{rec} and P_{dif} values that could fit all data sets: $P_{dif} = 2 \times 10^6$, $P_{rec} = 0.26$ (at 10 mM $MgCl_2$) and $P_{dif} = 1 \times 10^6$, $P_{rec} = 0.015$ (at 1 mM $MgCl_2$).

The intrinsic probabilities to recognize a specific site do not reflect the macroscopic situation, because after leaving a site by linear diffusion, the enzyme still has a high probability to reach this site for a second time. Simulations of this behavior have shown that a recognition efficiency of 0.26 means that about 75% of all enzymes will cleave the first recognition site that is encountered on the pathway of linear diffusion. Thus, macroscopically *Eco*RV efficiently recognizes cleavage sites if 10 mM $MgCl_2$ is present. However, the intrinsic probability of site recognition determined at low concentration of $MgCl_2$ (1 mM $MgCl_2$) is so small (0.015) that less than 10% of all enzymes will cleave the first site encountered during linear diffusion.

Monte Carlo simulations of the experiment shown in Figure 2 have demonstrated that under these conditions the enzyme on average will approach an end 220 times before it hits its recognition site. As blocking of the ends yields a rate enhancement of 2% at most, 220 associations to ends

will result in only 2% of the cases in a dissociation of the enzyme. This means that the probability of *Eco*RV to fall off at the end of a DNA molecule is below 10^{-4} (0.02/220). Using this value (P_{ref} = 9999/10 000) instead of the value of 1.0 used so far in the Monte Carlo simulation does not influence the results of the simulations shown in Figure 5. Thus, the probability of *Eco*RV to dissociate from linear DNA when reaching an end is so low that for purposes of kinetic analyses a model of complete reflection is applicable.

In the presence of 10 mM $MgCl_2$, the P_{dif} value obtained by the Monte Carlo simulations (2×10^6) does not deviate much from the value calculated using eq 1 (1.2×10^6). This is due to a fortuitous compensation of the systematic errors inherent in eq 1: On one hand, eq 1 underestimates the path length of linear diffusion because the enzyme does not always recognize its cleavage site. On the other hand, eq 1 overestimates the path length of linear diffusion because efficient end reflection leads to a shorter effective walking length than calculated by eq 1.[3] However, at 1 mM $MgCl_2$ both effects do not cancel each other: under this condition the P_{dif} value determined by simulation is much larger than that calculated by eq 1, because the efficiency of site recognition now is strongly reduced leading to a significant underestimation of the real path length of linear diffusion by eq 1. These results demonstrate that eq 1 does not adequately describe the kinetics of linear diffusion at a molecular level under conditions where recognition is not 100% efficient and/or end effects have to be considered.

Analysis of Processivity. It has been shown for *Eco*RI that dissociation of this enzyme from the cleavage products is preceded by a transfer of the protein to nonspecific DNA (*22*). This mechanism of product release causes a processive cleavage of two *Eco*RI sites located on the same substrate because a transfer of the enzyme to a cleavage product containing a second cleavage site leads to a very fast cleavage of this site. We have analyzed the cleavage rates determined in the experiments with the 32mer substrate 2sitesI (Figure 4) with respect to the processivity of *Eco*RV. The basic question in this analysis is whether the long cleavage product, which still contains an *Eco*RV cleavage site and is a kinetic intermediate (cf. Figure 1), is formed with a lower yield than expected for a distributive reaction. Such a result would indicate a processive cleavage of this substrate by *Eco*RV, implicating that product dissociation is preceded by linear diffusion of the enzyme to nonspecific DNA. As the substrate used in the experiments shown in Figure 4 is labeled on the 5′ end of the upper strand, only two cleavage products can be detected: the 5′-labeled short product (5′-SP) resulting from cleavage at site A and the 5′-labeled long product (5′-LP) resulting from cleavage at site B (cf. Figure 1). The relative rates of formation of these products depend on the relative cleavage rates at site A (k_{siteA}) and site B (k_{siteB}) as well as on the tendency of *Eco*RV to leave its cleavage sites via linear diffusion and thereby being transferred to an adjacent cleavage site (P_t) as given in eqs 2a and 2b. Clearly, the three unknown parameters of the system (k_{siteA}, k_{siteB}, and P_t) cannot be defined if only two properties are measured ($k_{5'-LP}$ and $k_{5'-SP}$). Therefore, a second set of experiments was carried out in which the upper strand of 2sitesI was labeled on its 3′ end and cleaved by *Eco*RV. In these experiments one short product (3′-SP) and one long product (3′-LP) can be observed, whose rates of formation are given by eqs 2c and 2d (cf. Figure 1).

$$k_{5'\text{-SP}} = k_{siteA} + 1/2P_t\, k_{siteB} \quad (2a)$$

$$k_{5'\text{-LP}} = k_{siteB} - 1/2P_t\, k_{siteB} \quad (2b)$$

$$k_{3'\text{-SP}} = k_{siteB} + 1/2P_t\, k_{siteA} \quad (2c)$$

$$k_{3'\text{-SP}} = k_{siteB} - 1/2P_t\, k_{siteA} \quad (2d)$$

with P_t as the probability of *Eco*RV to be transferred to nonspecific DNA after cleavage, $k_{5'-SP}$ as the initial rate of formation of the 5′-labeled short product, $k_{5'-LP}$ as the initial rate of formation of the 5′-labeled long product, $k_{3'-SP}$ as the initial rate of formation of the 3′-labeled short product, $k_{3'-LP}$ as the initial rate of formation of the 3′-labeled long product, k_{siteA} as the cleavage rate at site A, and k_{siteB} as the cleavage rate at site B. [The factor of 1/2 is introduced to correct for the fact that the enzyme can leave the site of cleavage in two directions, only one of which would target it to the second cleavage site. Note that eqs 2a−d only describe initial rates because the consecutive cleavage of the long products, 3′-LP and 5′-LP, unless occurring processively, is not considered.]

In the presence of 10 mM $MgCl_2$ and 50 mM NaCl, 5′-LP is formed 1.41 (±0.01) times faster than 5′-SP (Figure 4), and 3′-SP is formed 1.45 (±0.1) faster than 3′-LP (data not shown). On the basis of eqs 2a−d, these results imply that k_{siteB}/k_{siteA} = 1.43 (±0.05) and P_t = 0, indicating that under these conditions *Eco*RV does not processively cleave two recognition sites located on the same DNA piece. Consequently, product dissociation cannot involve a direct transfer to nonspecific DNA in the case of *Eco*RV. This conclusion is confirmed by our finding that site B is cleaved 4.9 times faster than site A in 2sitesII at 10 mM $MgCl_2$, a result that is not compatible with a processive cleavage of both sites.

DISCUSSION

In this study, we have investigated the kinetics of linear diffusion of *Eco*RV on DNA. We have shown that *Eco*RV is reflected at the ends of linear DNA molecules during this diffusional search. This behavior can be rationalized by the observations that *Eco*RV fully embraces the DNA in the protein−DNA complex (*46*) and that linear diffusion is strongly dependent on a balanced electrostatic interaction between *Eco*RV and the DNA (*12*). As the shape of the electrostatic potential at the ends of a linear DNA is fundamentally different from that in the middle of a long DNA piece, it might be difficult for the enzyme to approach a DNA end by linear diffusion. By analogy, a positively charged ring is unlikely to fall off a negatively charged stick. It will be interesting to see if *Eco*RV can *associate* to the

[3] In eq 1, association of *Eco*RV to the DNA can only occur on the sequence of the substrate. However, after association a random walk on an infinite lattice is assumed. Thus, if an end is encountered in the model described by eq 1, the enzyme can diffuse further on a virtual substrate whereas it is reflected in the real situation. Therefore, depending on the length of the substrates and the position of the cleavage site, eq 1 may overestimate the path length of linear diffusion significantly.

end of a linear DNA, thereby threading the DNA into its binding "tunnel"—as this simple physical model would predict. However, association over the ends cannot be involved in the major kinetic pathway of DNA cleavage because we have shown that blocking the ends does not change the cleavage rates.

During linear diffusion, *Eco*RV must scan the DNA and discriminate nonspecific from specific binding sites. After binding to a specific site, the enzyme might recognize this site or alternatively overlook it and continue with linear diffusion. One important result of this study is that the efficiency of DNA recognition, i.e., the probability that a specific site is recognized (and cleaved) after the enzyme has bound to it, is strongly dependent on the concentration of Mg^{2+}. If the Mg^{2+} concentration is low, the enzyme has a high probability to overlook a recognition site. Our results demonstrate that there is a continuous transition in the *Eco*RV−DNA interaction between hardly any binding specificity (absence of Mg^{2+}), low binding specificity (1 mM $MgCl_2$), and high binding specificity (10 mM $MgCl_2$). Consequently, the maximum range of linear diffusion is only 1000 bp at 1 mM $MgCl_2$ but >2000 bp at 10 mM $MgCl_2$. It should be mentioned that the transition between low and high specificity is observed between 1 and 10 mM Mg^{2+}, indicating that the affinity of the responsible metal ion binding site is in the millimolar range, in agreement with results of the Mg^{2+} concentration dependence of oligonucleotide cleavage rates (*56*). This result complements previous thermodynamic (*11, 48, 53, 54*) and kinetic data (*57*), which demonstrate that *Eco*RV does not bind to DNA with high specificity in the absence of Mg^{2+}. It is not easily reconciled with the assertion recently published by Engler et al. (*58*) that binding specificity of *Eco*RV is not dramatically enhanced by Mg^{2+}. However, the data given in this paper (*58*) only show a 30-fold preference of binding of *Eco*RV to specific sites over nonspecific sites at pH 7.0 (lowest specific binding constant to a 22mer, 1.8×10^8 M^{-1}; highest binding constant to a nonspecific 22mer, 6.4×10^6 M^{-1}).[4] It is not clear, how the authors, on the basis of their data, came to the conclusion that *Eco*RV binds to specific targets with a 12 000-fold preference. It should be noticed that a 30-fold preference of binding to a specific over binding to a nonspecific site would not at all be in disagreement with the results reported in our study, because *Eco*RV has a 10^5-10^6-fold binding specificity in the presence of Mg^{2+} (*48*), and one could easily imagine that a 10^4-fold change in the thermodynamics of DNA binding (specificity factor 30 vs 10^5-10^6) could cause the kinetic effect on linear diffusion observed here.

Although *Eco*RV is able to scan the DNA over several hundreds of base pairs in search of its recognition sequence, linear diffusion is not involved in product dissociation in *Eco*RV. Our data rather show that the enzyme directly dissociates from the cleavage products. This behavior is dissimilar to that of *Eco*RI where transfer to nonspecific DNA precedes dissociation of the enzyme from the reaction products (*50*). This distinction might be due to the different cleavage modes of *Eco*RV and *Eco*RI. Whereas *Eco*RV cleavage yields blunt ends (GAT↓ATC), *Eco*RI produces DNA fragments with a 4 bp overhang (G↓AATTC). Thus, it is conceivable that a DNA cleaved by *Eco*RI via its sticky ends retains an annealed double-stranded conformation when bound to the enzyme, thereby allowing *Eco*RI to leave the cleavage site by linear diffusion. In contrast, if the DNA is cleaved by *Eco*RV both products no longer are in physical contact to each other and might directly diffuse out of the DNA binding tunnel of the enzyme. This movement could be driven by the electrostatic repulsion between the DNA pieces that is further increased by the two additional negative charges produced at the newly formed 5′-phosphates upon cleavage.

A quantitative analysis using Monte Carlo simulations reveals that the ratio of rate constants of linear diffusion and dissociation (k_{dif}/k_{-1}) is 2×10^6 in 10 mM and 1×10^6 in 1 mM $MgCl_2$. It should be noticed that k_{dif}/k_{-1} is larger at 10 mM $MgCl_2$, although under these conditions the DNA binding constant is lower implicating that k_{-1} will be larger. One possible explanation for this finding could be that at low concentrations of $MgCl_2$ k_{dif} is smaller than at 10 mM $MgCl_2$. It resembles our observation that some *Eco*RV mutants that strongly bind to DNA have a largely reduced efficiency of linear diffusion (*12*). Possibly, too strong a DNA binding can interfere with linear diffusion and reduce k_{dif}, because a tight complex results in a larger friction that slows down the sliding movement.

The k_{dif}/k_{-1} value for linear diffusion of *Eco*RV determined at 10 mM Mg^{2+} (2×10^6) can be used to estimate the rate of linear diffusion (k_{dif}). Using the dissociation constant (k_{-1}) for *Eco*RV and macromolecular DNA of 1.2 s^{-1} determined in a buffer containing 10 mM $MgCl_2$ (*60*), the rate of linear diffusion (k_{dif}) can be estimated to be 1.7×10^6 s^{-1}, in agreement with estimates of Erskine et al. (*60*), who concluded from quenched flow experiments that the rate of linear diffusion must be higher than 5×10^5 s^{-1}. The rate constant of linear diffusion (k_{dif}) is related to the diffusion coefficient of Fick's law by $D_1 = 1/2k_{dif}\ l^2$, where l is the length of each step (0.34 nm in B-DNA). Using our data, a linear diffusion coefficient of $D_1 = 9.6 \times 10^{-14}$ m^2 s^{-1} can be derived that is close to the value obtained for *Eco*RI (*19*). Linear diffusion of *Eco*RI and *Eco*RV is by 2−3 orders of magnitude slower than three-dimensional diffusion, but the diffusion constant is close to its theoretical limit (given by hydrodynamics) of $D_1 = 5 \times 10^{-13}$ m^2 s^{-1} (*61*), a finding which stresses that restriction enzymes are optimized for this property during evolution.

Our data demonstrate that *Eco*RV on average occupies each nonspecific site not longer than for 1 μs. Nevertheless, the cleavage site is efficiently recognized if 10 mM $MgCl_2$ is present. Under these conditions *Eco*RV behaves like *Eco*RI (*24*) and *Bss*HII (*21*), which both do not overlook specific sites while diffusing along the DNA. The dynamics of the DNA recognition process by restriction endonucleases are illustrated by the finding that *Eco*RI pauses at star sites (i.e., sites that differ at only one position from the *Eco*RI recognition sequence) for several seconds. Pausing times are roughly correlated with the cleavage rates at these sites (*24*). Thus, the residence time at a certain DNA site appears

[4] This observation is in disagreement with data measured in several other laboratories (see, for example, refs *11*, *48*, *54*, and *59*). This discrepancy has led to new studies carried out by two groups who investigated the DNA-binding specificity of *Eco*RV in the absence of Mg^{2+} using a *homophasic* binding assay (fluorescence depolarization). In both cases, no evidence for specific binding of *Eco*RV to oligonucleotides was obtained (Erskine & Halford, submitted for publication; B. A. Connolly, personal communication).

to be important for coupling the formation of specific contacts between the restriction enzyme and the DNA (specificity) and catalysis. The more contacts are formed, the longer the residence time will be and the higher the probability of cleavage at a certain site. In agreement with this model it was found for *Eco*RI that the residence time at a cognate site is in the order of 15 min (*22*). One should note that, even if the probability of cleavage were similar at a nonspecific and a specific site, the difference in residence times at the sites could explain a preference of cleavage at specific sites (residence time ≈15 min) over nonspecific sites (residence time ≈1 μs) of 10^9. This degree of specificity approaches the discrimination observed with restriction enzymes between truly nonspecific sites and recognition sites, because sites differing from the recognition sequence in more than one base pair are not cleaved to a detectable extent. This model would predict that star sites (residence time ≈1 s) are cleaved approximately 3 orders of magnitude more slowly than specific sites, which is in excellent agreement with results of oligonucleotide cleavage experiments carried out with *Eco*RI (*51*, *52*) and *Eco*RV (*53*). However, both *Eco*RI and *Eco*RV usually cleave star sites only in one DNA strand, whereas the specific site is cut in both strands indicating that the mode of cleavage of a star site is different from that of a specific site in both enzymes, which is probably mediated by cooperative conformational changes of the enzyme−DNA complex (reviewed in ref *5*).

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