



Diffusion of the Restriction Nuclease EcoRI along DNA

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Many specific sequence DNA binding proteins locate their target sequence by first binding to DNA nonspecifically, then by linearly diffusing or hopping along DNA until either the protein dissociates from the DNA or it finds the recognition sequence. We have devised a method for measuring one-dimensional diffusion along DNA based on the ratio of the dissociation rate of protein from DNA fragments containing one specific binding site to the dissociation rate from DNA fragments containing two specific binding sites. Our extensive measurements of dissociation rates and specific–nonspecific relative binding constants of the restriction nuclease EcoRI enable us to determine the diffusion rate of nonspecifically bound protein along the DNA. By varying the distance between the two binding sites, we confirm a linear diffusion mechanism. The sliding rate is relatively insensitive to salt concentration and osmotic pressure, indicating that the protein moves smoothly along the DNA probably following the helical phosphate-sugar backbone of DNA. We calculate a diffusion coefficient for EcoRI of $3 \times 10^4 \text{ bp}^2 \text{ s}^{-1}$. EcoRI is able to diffuse $\sim 150 \text{ bp}$, on average, along the DNA in 1 s. This diffusion rate is about 2000-fold slower than the diffusion of free protein in solution. A factor of 40–50 can be accounted for by rotational friction resulting from following the helical path of the DNA backbone. Two possibilities could account for the remaining activation energy: salt bridges between the DNA and the protein are transiently broken, or the water structure at the protein–DNA interface is disrupted as the two surfaces move past each other.

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Introduction

The initial observation that the association rate of *lac* repressor binding to its operator DNA was significantly faster than the diffusion limited rate led to the realization that association occurs in two steps.^{1–6} Many sequence-specific DNA binding proteins will initially bind nonspecifically to DNA and subsequently move along the DNA helix until either the protein finds a recognition sequence or the protein dissociates. This combination of one-dimensional and three-dimensional searching can lead to association rates that are much faster than expected for only a three-dimensional search,^{1,7–9} although whether experimentally observed rates are faster due to one-dimensional searching has been recently questioned.¹⁰ After nonspecific association, the extent of DNA that the protein can

explore depends on the rates of both diffusion along the DNA and dissociation of the nonspecifically bound complex. Initially, estimates of the length of DNA explored have come from the sensitivity of the association rates to DNA length.⁶ The ability of restriction endonucleases to cleave DNA after finding their recognition sequences has proven particularly useful for measuring the DNA-length-dependent rates of association.^{11–14} These rates depend on the rates of the protein's association with and dissociation from nonspecific DNA and on one-dimensional diffusion coefficients. Determining the encounter rates between free protein and DNA of different lengths and the dissociation rate of nonspecifically bound complex can, however, be problematic.

Recently, there have been several direct single-molecule measurements of one-dimensional diffusion rates using fluorescently labeled protein.^{2,15–21} This approach avoids the problems inherent in knowing the association and nonspecific complex dissociation rates. The protein must stay on the

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DNA long enough and move far enough for a precise measurement.

Three mechanisms for the movement of proteins along DNA have been postulated.³ Sliding refers to base-pair-by-base-pair diffusion along DNA; the protein remains in contact with the helix. Several crystal and NMR structures of nonspecific DNA–protein complexes (e.g., Viadiu and Aggarwal,²² Kalodimos *et al.*,²³ and Winkler *et al.*²⁴) and our own measurements of the difference in sensitivity to water activity between specific and nonspecific binding modes for several DNA–protein complexes^{25–28} show that substantial water is present at the DNA–protein interface of nonspecific complexes which can ‘lubricate’ movement along the helix.²² Hopping is the transient dissociation and rebinding of the protein to the same helix.^{29,30} Hopping would be expected to have significant salt dependence, since transient dissociation breaks DNA–protein charge interactions. Hopping can span from a few base pairs to many base pairs in one movement. Direct transfer is the reaction of free DNA with a complex and consequent exchange of the protein from one helix to another.

Our approach here is to measure the ratio of the specific site dissociation rate of a DNA fragment with a single EcoRI binding site to the specific site dissociation rate of another fragment with two binding sites separated by variable lengths. The probability of reaching the second site after dissociation from the first site affects the dissociation rate of the two-site DNA fragment relative that of the one-site DNA fragment. By measuring the ratios of dissociation rates, we eliminate the transition kinetics between specific and nonspecific binding modes at the recognition site. In order to better fit the one-dimensional diffusion rate, we vary the nonspecific complex dissociation rate over an 8000-fold range by varying the salt concentration. The overall specific site dissociation rate is held approximately constant by appropriately adjusting the osmotic pressure that varies the transition kinetics between the specific and nonspecific binding modes at the specific site. We use our previous measurements³¹ of the nonspecific complex dissociation rate to calculate the sliding rate or the one-dimensional diffusion coefficient from fits to the data using the theoretical framework of Belotserkovskii and Zarling.³² Our results also indicate that the sliding rate is insensitive to salt concentration and osmotic pressure, suggesting that EcoRI follows the helical backbone as it diffuses. We find that the sliding rate of EcoRI along DNA is about 2000-fold slower than expected if only translational flow friction is considered. This is consistent with measurements on several other DNA–protein complexes. At least part of the extra friction can likely be attributed to rotational flow friction, as the protein follows the helical path of DNA. The remainder is perhaps due to transient breaking and remaking of DNA–protein charge pairs or to disruption of water structuring at the protein–DNA interface.

Theory

We use the theoretical framework described by Belotserkovskii and Zarling for random walks, with a probability for irreversible dissociation.³² Probabilities—not rates—of dissociation are calculated. However, we work under conditions where the dissociation rate of a nonspecifically bound complex is much faster than the transition between specific and nonspecific binding modes (at any time, most complexes are specific). We previously measured the nonspecific dissociation rate for an oligonucleotide as 50 min^{−1} at 90 mM NaCl (pH 7.0) and 20 °C, whereas the dissociation rate of a specific site oligonucleotide complex under the same experimental conditions was ~0.005 min^{−1}.^{31,33} This means that ratios of dissociation probabilities, once the protein is nonspecifically bound, can substitute for ratios of rates.

Within the Belotserkovskii and Zarling framework, the parameters k_{sl} and $k_{nsp,diss}$ are the rate constants for moving 1 bp along the DNA (equally probable in either direction) and for dissociation of the nonspecifically bound complex, respectively. The dissociation rate constant from the ends $k'_{nsp,diss}$ may be different from interior sequences. We assume that the specific recognition sites are absorbing barriers and that the random walk begins 1 bp from a binding site.

The probability that a protein starting next to a binding site and with an end in the opposite direction 1 bp away will rebind before dissociating can be calculated as:

$$P_1(l) = e^{-\sqrt{a}} \frac{(1+c) + (1-c)e^{-2\sqrt{a}(l-2)}}{(1+c) + (1-c)e^{-2\sqrt{a}(l-1)}} \quad (1)$$

where

$$a = \frac{k_{nsp,diss}}{k_{sl}} \text{ and } c = \sqrt{a} \left(\frac{k'_{nsp,diss}}{k_{nsp,diss}} \right) \quad (2)$$

The probability that a protein starting next to a binding site (with another binding site in opposite direction to the first and ΔL base pairs away) will find one or the other binding sites before dissociating is:

$$P_2(\Delta L) = \frac{\sinh(\sqrt{a}) + \sinh(\sqrt{a}(\Delta L - 1))}{\sinh(\sqrt{a}\Delta L)} \quad (3)$$

The dissociation probability for the single-site fragment with distances l_1 and l_2 base pairs from the binding site to the ends is:

$$P_{diss,1-site}(l_1, l_2) = 1 - P_1(l_1) / 2 - P_1(l_2) / 2 \quad (4)$$

Similarly, the dissociation probability for the two-site fragment with l_3 base pairs from the end to the first binding site, ΔL base pairs between sites, and

l_4 base pairs between the second binding site and the far end is:

$$P_{\text{diss},2\text{-site}}(l_3, \Delta L, l_4) = 1 - P_1(l_3) / 4 - P_2(\Delta L) / 2 - P_1(l_4) / 4 \quad (5)$$

The ratio of specific complex off-rates is then given by:

$$\frac{P_{\text{diss},1\text{-site}}}{P_{\text{diss},2\text{-site}}} = \frac{1 - P_1(l_1) / 2 - P_1(l_2) / 2}{1 - P_1(l_3) / 4 - P_2(\Delta L) / 2 - P_1(l_4) / 4} \quad (6)$$

For the DNA fragments used and for the $k_{\text{nsp,diss}}$ values examined, this ratio depends primarily on the distance ΔL between sites and the ratio of nonspecific complex dissociation to sliding rates through the parameter a (Eq. (2)); the parameter c (Eq. (2)) affects fits to the data minimally for the DNA fragments used (a value of $c=1$ will be assumed for the initial fits to data with 360-bp DNA fragments). A better value will be estimated from a comparison of specific site dissociation rates for 360-bp fragments and 30-bp oligonucleotides.

Results

Figure 1 illustrates the basic principle in comparing the dissociation rates of DNA fragments containing one and two specific binding sites. The initial step is a transition between specific and nonspecific

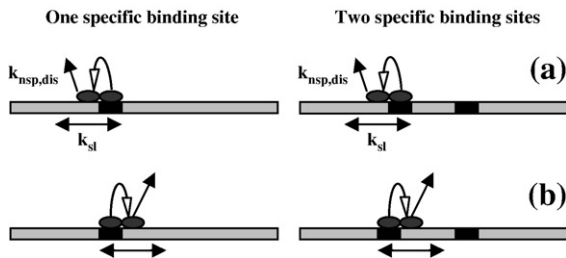


Fig. 1. A schematic representation illustrating the basis for the method used for measuring sliding rates. The dissociation reaction starts with a transition from specific to nonspecific binding modes at the recognition sites (black boxes), followed by a sliding translation to either left (a) or right (b). If the recognition sites are considered absorbing barriers and if the walks start from the left, then the probabilities that EcoRI will dissociate from one-site and two-site DNA fragments are the same. Starting from the right, however, probabilities will be the same only if the dissociation rate is much faster than the linear diffusion rate. If the sliding rate is much faster than the dissociation rate, the ratio of the dissociation probability for the two-site DNA fragment to the dissociation probability for the one-site DNA fragment is 0. The protein will find the second site before dissociating. The ratio of the off-rates for complexes with the one-site fragment to the off-rates for complexes with the two-site fragment will, therefore, vary between 2 and 1, depending on the ratio of the nonspecific complex dissociation rate to the sliding rate.

binding modes. The nonspecifically bound protein then begins a random walk and can either dissociate, return to the original binding site, or diffuse to the second binding site for the two-site fragment. If the protein begins the random walk to the left and if binding sites are absorbing barriers, then the complexes with one and two binding sites have equal probabilities of either dissociating from the DNA or returning to the original binding site. If, however, the protein begins the random walk to the right such that it is bracketed by the recognition sequences of the two-site fragment, then the dissociation rates of the two fragments are not necessarily the same. If the nonspecific dissociation rate $k_{\text{diss,nsp}}$ is much faster than the one-dimensional diffusion rate k_{sl} , then the probability that the protein will reach the other site before dissociating is quite small. The dissociation rates of the one-site and two-site DNA fragments are once again the same. If, however, the one-dimensional diffusion rate is much faster than nonspecific dissociation, then the probability that the protein will reach the other site before dissociating is very high. In this case, the two-site fragment will dissociate half as fast as the one-site fragment; only those complexes that begin the random walk to the left will have a chance of dissociating. The ratio of the one-site dissociation rate to the two-site dissociation rate will vary between 1 and 2, depending on the relative rates of one-dimensional diffusion and nonspecific complex dissociation. The equations describing the dependence of the ratio of the dissociation rate constants on k_{sl} and $k_{\text{nsp,diss}}$ are given in Theory.

The nonspecific dissociation rate can be controlled such that the probability of reaching the second site can be varied, allowing a better fit to the equations. We have extensive measurements of the osmotic pressure and salt concentration dependence of the relative specific-nonspecific binding constant and the specific site dissociation rate from previous work. The fundamental Gibbs-Duhem equation relates the dependence on osmotic pressure $d \ln(K)/d\Pi$ or $d \ln(k)/d\Pi$ to the difference in the number of sequestered or bound water molecules between the initial state and the final or transition state, while the salt sensitivity $d \ln(K)/d \ln[\text{salt}]$ or $d \ln(k)/d \ln[\text{salt}]$ primarily gives a difference in the number of thermodynamically bound ions.³⁴ We have found that these dependencies are linear over a wide range of salt concentrations and betaine osmotic pressures for the relative specific-nonspecific binding constant and the specific site dissociation rate.^{31,33} The relative specific-nonspecific binding constant and the specific site dissociation rate have about the same large osmotic pressure dependence, but the specific site dissociation rate has much greater salt concentration sensitivity than the relative binding constant. As in Fig. 1, the dissociation rate of EcoRI from a specific site DNA fragment can be divided into two processes: a transition between specific and nonspecific binding modes that will have the same osmotic pressure and salt concentration dependences as the relative

specific–nonspecific binding constant and a dissociation of nonspecifically bound protein. We assume that the difference in osmotic and salt dependencies between the relative specific–nonspecific binding constant and the specific sequence dissociation rate is the difference in sequestered water and salt binding between the nonspecific complex and the dissociation transition state of the nonspecific complex (i.e., the osmotic and salt sensitivity of $k_{\text{nsp,diss}}$). Therefore, we can vary $k_{\text{nsp,diss}}$ widely through the salt concentration, but maintain a conveniently measured overall specific site off-rate by adjusting the osmotic pressure to control the equilibrium between specific and nonspecific binding. $k_{\text{nsp,diss}}$ at any salt concentration (in mM) and glycine betaine osmotic pressure (in osmolal) can be related to $k_{\text{nsp,diss}}^0$ at 90 mM NaCl and 0 osmolal glycine betaine (20 °C and pH 7.0) by:

$$\ln(k_{\text{nsp,diss}} / k_{\text{nsp,diss}}^0) = 7.6 \times \ln([\text{NaCl}] / 90) - 0.3 \times \text{osmolal} \quad (7)$$

The parameter a of Eq. (2) and, ultimately, of Eq. (6) can be rewritten in terms of the ratio of nonspecific complex dissociation rates $k_{\text{nsp,diss}}/k_{\text{nsp,diss}}^0$ as:

$$a = \frac{k_{\text{nsp,diss}}}{k_{\text{sl}}} = \left(\frac{k_{\text{nsp,diss}}^0}{k_{\text{sl}}} \right) \left(\frac{k_{\text{nsp,diss}}}{k_{\text{nsp,diss}}^0} \right) \quad (8)$$

The only fitting parameter in Eq. (6) is then ($k_{\text{nsp,diss}}^0/k_{\text{sl}}$).

Figure 2a shows a gel mobility shift assay for measuring dissociation rates. At time $t=0$, an oligonucleotide containing the *EcoRI* recognition sequence is added to the reaction mixture of protein and DNA fragment in large excess. The molar ratio of oligonucleotide to DNA fragment is typically ~ 500 . The protein that dissociates is efficiently removed from further reaction with the DNA fragment. The experimental conditions (90 mM NaCl, pH 7.0, and 20 °C, with no added Mg^{2+}) are such that *EcoRI* binds essentially stoichiometrically to DNA. The dissociation reaction is stopped by adding triethylene glycol, which both lowers the salt concentration and greatly increases the osmotic pressure, both of which substantially decrease (by more than 100-fold) the specific site dissociation rate. The five lanes on the left illustrate the increasing loss of the complex with increasing time of incubation with oligonucleotide for the one-site fragment. The five lanes on the right show the corresponding dissociation of the protein from the two-site fragment with 21 bp between sites ($\Delta 21$). It is apparent that the two-site fragment complex dissociates more slowly. The two-site fragment shows a small fraction of fragment with two bound proteins.

Figure 2b shows the semilog plots for the dissociation kinetics of complexes with one bound protein for the one-site and two-site fragments shown in Fig. 2a. The kinetics for the two-site fragment can be corrected for the dissociation of the complex with

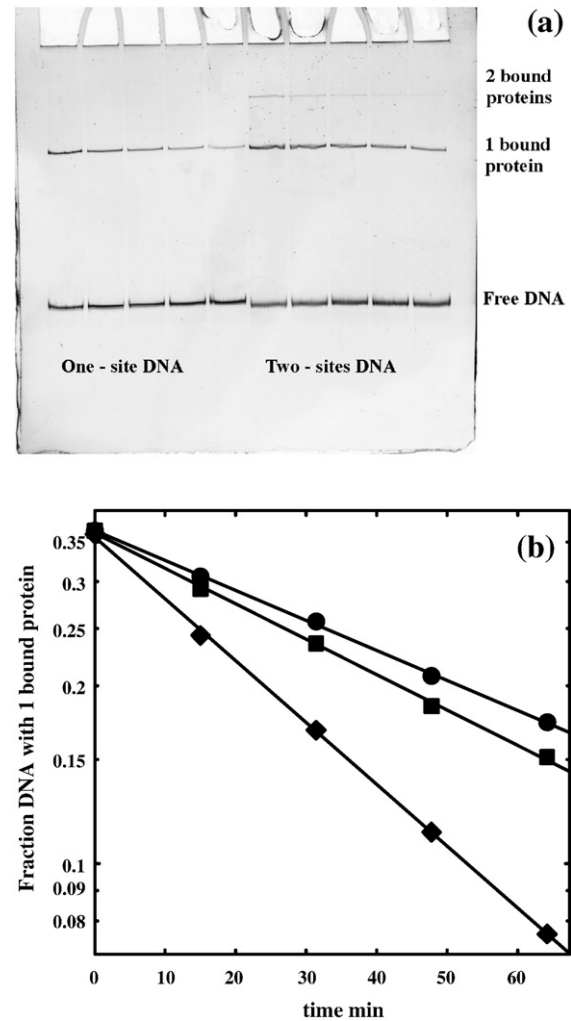


Fig. 2. Dissociation of *EcoRI* from DNA with one or two recognition sites. (a) A typical polyacrylamide gel shows the loss of *EcoRI* binding to the one-site DNA fragment (the first five lanes) with time and to the two-site DNA fragment (the last five lanes). The one-protein bound complex and free DNA bands are indicated. The two-site DNA fragment has an additional weak band of complex with two bound proteins. A large excess of specific site oligonucleotide is added to the *EcoRI*/DNA fragment mixture to initiate dissociation. Incubation times are 0, 15, 30, 45, and 60 min from left to right. The dissociation reaction is stopped by adding triethylene glycol. (b) The logarithmic dependences of the fraction of one bound protein complex on time are shown for the one-site (◆) and two-site (●) DNA fragments shown in (a). The slopes give the off-rates for *EcoRI* dissociating from the fragments into solution $k_{\text{off,1-site}}$ or $k_{\text{off,2-site}}$. A correction to the fraction of one bound protein complex, to account for the contribution of the dissociation of the two-site fragment with two bound proteins, can be applied using Eq. (9). The corrected data are shown (■). The observed ratio $k_{\text{off,1-site}}/k_{\text{off,2-site}} = 1.93$ indicates that the *EcoRI* that dissociates from one specific site and begins a random walk in the direction of the other site will almost certainly find the other before dissociating from the fragment into solution (Fig. 1b).

two bound proteins, as described by Eq. (9). The ratio of the one-site dissociation rate to the two-site dissociation rate is 1.9, indicating that the protein is able to diffuse the 21-bp separation between sites with high probability before dissociating.

Figure 3 shows the ratio of one-site dissociation rate to two-site dissociation rate for two-site separations of 21, 26, and 84 bp as a function of the normalized nonspecific dissociation rate $\ln(k_{\text{nsp,diss}}^0/k_{\text{nsp,diss}})$, as the salt and osmotic pressures are varied (Eq. (7)). The ratios do vary between 1 and 2, as predicted. The continuous lines are fits of the data to Eq. (6), with $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}}) = -12.6$. At $k_{\text{diss,1-site}}/k_{\text{diss,2-site}} = 1.5$, the nonspecific dissociation rate is about 16-fold faster for $\Delta 21$ two-site separation than for the $\Delta 84$ two-site fragment, as would be expected for simple diffusion and the 4-fold difference in distance between sites. The best fit of Eq. (6) to the 21-

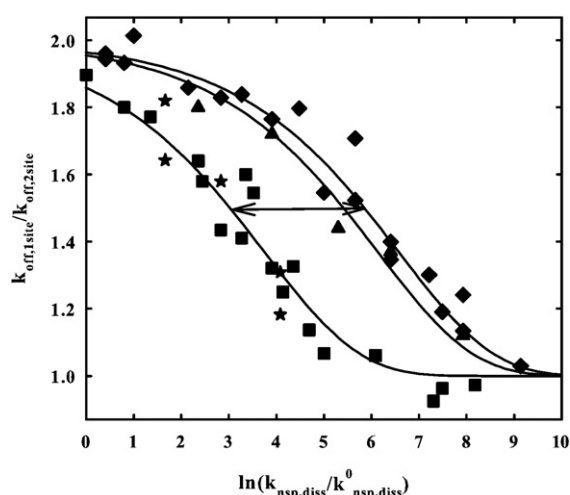


Fig. 3. The ratio of one-site fragment dissociation rate constants to two-site fragment dissociation rate constants determined in Fig. 2 is shown as a function of $\ln(k_{\text{nsp,diss}}^0/k_{\text{nsp,diss}})$; $k_{\text{nsp,diss}}^0$ is the nonspecific complex dissociation rate at 90 mM NaCl, 0 Osm, pH 7.0, and 20 °C, and $k_{\text{nsp,diss}}$ is the nonspecific complex dissociation rate at the salt and osmolyte concentrations used. From our previous data, $\ln(k_{\text{nsp,diss}}^0/k_{\text{nsp,diss}})$ is given by Eq. (7). The salt concentration is varied from 90 to 360 mM NaCl, and the betaine glycine osmolyte concentration is varied from 0 to 4 Osm to give the range examined. The ratios of the dissociation rates for the fragment with two sites separated by 84 bp are given by (■). The 'star' (★) data points for the $\Delta 84$ bp two-site fragment were obtained by keeping the osmolal concentration of betaine glycine constant and only varying the salt concentration. Dissociation rate ratios for the $\Delta 21$ bp two-site fragment are shown by (◆), and dissociation rate ratios for the $\Delta 26$ bp two-site fragment are shown by (▲). The two sites are on the same side of the DNA helix for $\Delta 84$ and $\Delta 21$, but on opposite sides for $\Delta 26$. The continuous lines are calculations of Eq. (6) with $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}}) = -12.6$. The arrow represents a 16-fold change in $k_{\text{nsp,diss}}$ for the 4-fold change in spacing between sites, as expected for a diffusion process. A complete data set that includes osmolal and salt concentrations, $k_{\text{off,1-site}}$ and $k_{\text{off,2-site}}$, as well as a graph of fitting errors, is given in [Supplementary Materials](#) for the $\Delta 21$ and $\Delta 84$ two-site DNA fragments.

bp spacing data alone is $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}}) = -12.7 \pm 0.2$, and the best fit to the $\Delta 84$ -bp distance data alone is $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}}) = -12.6 \pm 0.2$. There is not enough data to fit the $\Delta 26$ -bp spacing data independently and accurately. The good fit of the continuous line to the $\Delta 26$ -bp spacing data with $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}}) = -12.6$, however, indicates that it is not important if the recognition sites of the two-site DNA are on the same side or on the opposite side of the helix. The second recognition site is found with the same probability after dissociation from the first. The 'star' points in the $\Delta 84$ two-site fragment data show the result of only varying the salt concentration while holding the osmotic pressure constant. The points lie on the same curve within experimental error.

Discussion

The one-dimensional movement of nonspecifically bound protein along DNA can greatly accelerate specific site association kinetics. There have been several theoretical estimates of the optimal combination of one-dimensional and three-dimensional diffusions.^{7–9} This, of course, depends sensitively on the one-dimensional diffusion rate. In the cell, so many proteins are bound to DNA, blocking linear movement such that only small lengths of DNA are essentially accessible for scanning. There is no advantage to sliding ranges of thousands of base pairs.

A wide range of one-dimensional diffusion coefficients of proteins moving along the DNA have been measured using single-molecule techniques. Some, such as the DNA repair enzyme oxoguanine DNA glycosylase 1¹⁹ and p53,²⁰ have one-dimensional diffusion coefficients almost as large as the three-dimensional diffusion coefficients in dilute solution. There are only small additional energy barriers to movement along the DNA. Others, such as *EcoRV*,¹⁵ the *LacI* repressor,¹⁷ and the β -clamp,¹⁶ move significantly more slowly (at least 1000-fold) along DNA than free in solution, indicating significant energy barriers.

In addition to one-dimensional diffusion or sliding, other mechanisms commonly considered include 'hopping' and direct DNA-to-DNA transfer. Hopping is the dissociation and rebinding of protein to the same DNA (i.e., dissociation is only transient).³ Direct transfer is due to the collision of a protein–DNA complex with DNA either on the same fragment or on another fragment.³ The protein is transferred from the first DNA site to the second. By using high concentrations of specific site oligonucleotides to act as a sink for dissociated proteins, we accentuate the contribution from one-dimensional sliding. The specific site dissociation rate does not depend on the oligonucleotide concentration added over a 100–1000 range of ratios between specific site concentrations of oligonucleotide and DNA fragment. This indicates that direct transfer does not contribute significantly to our measurements of movement along DNA compared with one-dimen-

sional diffusion. It would also strongly suggest that the contribution from hopping is not significant.

Many DNA binding proteins are not highly stringent for the specific recognition sequence, but can bind to related noncognate sequences more strongly than to completely nonspecific sequences. The sliding rate at a particular site is likely related to the binding constant at that site. Sliding rates could be strongly sequence dependent. In this respect, the binding specificity of EcoRI is extraordinary. In 90 mM NaCl and at pH 7.0 and 20 °C, the ratio of equilibrium constants for binding to a specific recognition sequence and to a nonspecific 30-bp oligonucleotide^{25,31} is $\sim 2 \times 10^4$. A 30-bp oligonucleotide containing the 'star' sequence TAATTC that differs by a single base pair from the recognition sequence binds EcoRI only 2-fold more strongly than the nonspecific oligonucleotide.³¹ Other 'star' sequences with single base-pair changes from the recognition sequence bind even more weakly.³⁵ Sequences with two or more base-pair changes from the recognition sequence are virtually indistinguishable from nonspecific DNA.³⁵ The DNA fragments we use here do not contain any 'star' sequences between the two sites. We expect that EcoRI sliding rates under our experimental conditions are not particularly sequence dependent.

The ratio of specific site dissociation rates of complexes with the one-site the two-site DNA fragments, with $\Delta L = 84, 26$, and 21 bp shown in Fig. 3, can all be well fitted by Eq. (6) with $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}}) = -12.6 \pm 0.2$. This would indicate that the assumptions underlying the equations are correct. It was assumed that a recognition site is an absorbing barrier (i.e., that the protein does not pass over a site or miss a site). It does not matter if the site is on the same side ($\Delta 21$) or on the opposite side ($\Delta 26$) of the helix; EcoRI has the same high probability of finding the site if the protein diffuses to it. This also indicates that the protein does not transiently dissociate and take hops of several base pairs on the DNA. Equation (6) also assumes that k_{sl} does not have a significant salt concentration or osmotic pressure dependence. The salt concentrations and osmotic pressures are changed significantly over the range of $\ln(k_{\text{nsp,diss}}^0/k_{\text{nsp,diss}})$ from 1 to 9 (an 8000-fold change in rate) in Fig. 3. The salt concentration was varied from 90 to 360 mM, and the osmotic pressure (to maintain an approximately constant k_{off}) was varied from 0 to 4 Osm. The data fits then indicate that k_{sl} does not depend significantly on salt concentration or osmotic pressure, suggesting that the motion along the helix maintains approximately the same cushion of water at the protein–DNA interface and remains in close contact with the DNA-phosphate backbone. This would be consistent with previous suggestions that the nonspecifically bound protein follows the helical path of DNA. In agreement with the EcoRI results here, the one-dimensional diffusion coefficients of oxoguanine DNA glycosylase 1¹⁹ and p53²⁰ have little salt concentration sensitivity. A nonnegligible salt concentration dependence has been reported, however, for EcoRV sliding¹⁵ with added Mg^{2+} .

We have previously estimated the nonspecific complex dissociation rate $k_{\text{nsp,diss}}^0$ from the effect of added nonspecific 30-bp oligonucleotides on the specific sequence association rate under conditions of little free protein.⁹ The protein was either bound to the specific sequence fragment or bound to nonspecific oligonucleotide. Under standard conditions (0 Osm, 90 mM salt, 20 °C, and pH 7.0), $k_{\text{nsp,diss}}^0 = 0.8 \text{ s}^{-1}$. The ratio of this value to $k_{\text{sp,diss}}^0$ for a 30-bp specific site oligonucleotide was consistent with our previously measured value for the ratio of specific site binding constant to nonspecific site binding constant. The association and dissociation rates from the 30-bp specific site oligonucleotide are both ~ 4 -fold slower than for the 360-bp fragments, reflecting the contribution of ends to dissociation kinetics. Dissociation rates from DNA ends can be much different from internal sequences. We can calculate the ratio of the dissociation rate for the specific site 30-bp DNA fragment to the dissociation rate for the 360-bp DNA fragment varying the relative dissociation rate from the ends using the formalism of Belotserkovskii and Zarling and Eq. (4). If there was no difference in the dissociation rates from the internal sequences and the end sequences ($c=1$ in Eq. (2)), the 30-bp oligonucleotide off-rate is calculated to be ~ 11 -fold slower than that of the 360-bp fragment using the $k_{\text{nsp,diss}}^0/k_{\text{sl}}$ ratio determined from the fit to Fig. 3. In order to account for the observed ratio of 4, the dissociation rate would have to be ~ 40 -fold faster from the ends than from internal sequences. The dissociation rate of the 30-bp nonspecific oligonucleotide can be corrected for end dissociation to give $k_{\text{nsp,diss}}^0 = 0.2 \text{ s}^{-1}$. Including a 40-fold difference between ends and internal sequences in the calculation of the ratio of one-site dissociation rate to two-site dissociation rate for the 360-bp DNA fragments does not significantly change the fits shown in Fig. 3 or the best-fitting value of $\ln(k_{\text{nsp,diss}}^0/k_{\text{sl}})$.

With $k_{\text{nsp,diss}}^0 = 0.2 \text{ s}^{-1}$, we calculate that $k_{\text{sl}} = 6.0 \times 10^4 \text{ bp}^2/\text{s}$, corresponding to a diffusion rate of $\sim 3.5 \times 10^{-11} \text{ cm}^2/\text{s}$ along the helix. Under standard conditions of 90 mM salt, 20 °C, and pH 7.0 in the absence of Mg^{2+} , EcoRI can scan ~ 400 bp, on average, before dissociating. The diffusion coefficient is ~ 2000 -fold smaller than expected if the translational frictional drag of the solution is limiting, similar to the results for EcoRV. If proteins follow the helical path of DNA, as seems likely for EcoRI, then at least part of the extra friction comes from rotational motion, as first pointed out by Schurr³⁶ and further developed by Bagchi *et al.*³⁷ With the use of the latter, rotational friction slows the movement of EcoRI along the DNA by a factor of 40–50, leaving only an additional factor of 40–50 to account for. This is somewhat surprising given the ~ 10 charge–charge interactions between DNA and protein in the nonspecific complex. If the remaining factor of 40–50 is due to the breaking and remaking of these DNA–protein 'salt bridges,' the activation energy barrier would only be $\sim 0.4 \text{ kT}$ ($\sim 250 \text{ cal}$) for each. Additionally, the 110 waters at the DNA–protein

interface could be structured, with an energy penalty for perturbing this structuring as the protein moves.

As a final check on the measured sliding rate, we can also estimate the absolute dissociation rate constant of the 360-bp single specific site DNA fragment. Figure 4 illustrates the steps involved in arriving at the initial state considered in Fig. 1, with the protein bound nonspecifically next to the specific site. There is first a transition between specific and nonspecific binding modes at the specific site, followed by a sliding step in either direction. Since the nonspecific binding mode has ~ 110 water molecules sequestered at the DNA-protein interface, we do not expect the nonspecific mode binding constant to have a significant dependence on sequence. If the rate of returning to the specific binding mode is much faster than the sliding rate, as seems likely, then the rate of formation of the initial state is $2k_{sl}K_{sp-nsp}$, where K_{sp-nsp} is the ratio of nonspecific and specific site association constants. The overall dissociation rate is then $2k_{sl}K_{sp-nsp}P_{diss,1-site}$, where $P_{diss,1-site}$ is the probability that the protein will dissociate before rebinding to the specific site and is given by Eq. (4). Under our standard experimental conditions (pH 7.0 and 90 mM NaCl), the calculated $P_{diss,1-site}$ is 7×10^{-4} for $\ln(k_{nsp,diss}/k_{sl}) = -12.6$. As mentioned above, the relative binding constant for the competition between a 360-bp specific site fragment and a 30-bp nonspecific oligonucleotide is $\sim 1/(2 \times 10^4)$. If we consider that the 30-bp nonspecific oligonucleotide contains ~ 25 possible sites, then the K_{sp-nsp} for each nonspecific site is $\sim 0.5 \times 10^{-4}/25$. The specific site dissociation rate constant k_{off} for the 360-bp specific site fragment can then be estimated as $\sim 1.7 \times 10^{-4} \text{ s}^{-1}$, compared with our measured value of $\sim 2 \times 10^{-4} \text{ s}^{-1}$. The agreement is quite reasonable and supports our analysis.

Conclusions

By measuring the ratio of the dissociation rate of a DNA-protein complex with one specific recognition binding site to the dissociation rate of a complex with two specific recognition sites, we are able to determine the ratio of the nonspecific complex dissociation rate to the linear diffusion rate. At 90 mM NaCl, pH 7.0, and 20 °C, EcoRI is able to

cover ~ 400 bp, on average, before dissociating. The sliding rate is insensitive to salt and osmolyte concentrations, suggesting that the protein follows the sugar-phosphate backbone of DNA. The diffusion rate is about 2000-fold slower than the diffusion of free protein in solution. A factor of 40–50 can be accounted for by a rotational flow friction if the protein follows the helical path of DNA. The rest is likely due to the transient disruption of either DNA-protein charge interactions or water structuring at the protein-DNA interface.

Materials and Methods

Materials

Betaine glycine and triethylene glycol were purchased from Fluka Chemical Co. and used without further purification.

The DNA fragment containing one EcoRI site was prepared from pNEB193 (New England Biolabs), as described below. DNA fragments with two EcoRI sites were constructed by inserting oligonucleotides into pNEB193 cleaved in the polylinker region at two restriction endonuclease sites. The sequence of the oligonucleotide used to construct the two-site DNA with a 21-bp separation between sites ($\Delta 21$) was CAGTGAATTCGAGCGCGGGG. It was inserted into the KpnI/BamHI gap of cleaved pNEB193. The sequence of $\Delta 26$ two-site oligonucleotide was CGGGCCAGTGAATTCGAGCG and was also inserted into the KpnI/BamHI gap. The $\Delta 84$ two-site DNA was constructed with the oligonucleotide GGCGAGGGCCAGTGAATTCG, which was inserted into the PstI/HindIII gap. The EcoRI recognition sites are underlined. The second EcoRI sites have the same 3-bp flanking sequences on either side of the site as the native EcoRI site in pNEB193 to ensure that both sites bind EcoRI equally well. *Escherichia coli* (New England Biolabs turbo-competent cells) was transformed with cloned plasmids and individual colonies containing the correct plasmids isolated using standard techniques. Plasmids were isolated from 500-ml cultures using the Sigma GenElute HP Plasmid Megaprep kit. The one-site and two-site plasmids were then digested with PvuII, and the 360-bp fragments were isolated from an agarose gel during electrophoresis. The fragments were then amplified using standard PCR protocols.

The specific sequence oligonucleotide had been described by Sidorova and Rau.³¹ Purified EcoRI was a kind gift from Dr. L. Jen-Jacobson.

Dissociation reaction

The dissociation reaction mixture contained 20 mM imidazole (pH 7.0), 1 mM EDTA, 2 mM DTT, and 100 $\mu\text{g/ml}$ acetylated bovine serum albumin (Ambion). The NaCl concentration varied between 90 and 360 mM, and the betaine glycine concentration varied between 0 and 4 Osm (0–24% wt/wt). The DNA fragment concentration was 2 nM, and the EcoRI concentration was ~ 0.9 nM. The enzyme was allowed to equilibrate with the DNA fragment for ~ 30 min at 20 °C. The dissociation reaction was initiated by adding oligonucleotide containing the recognition site for EcoRI to a final concentration of 1 μM . The total reaction volume was 30 μl . The dissociation rate was insensitive to oligonu-

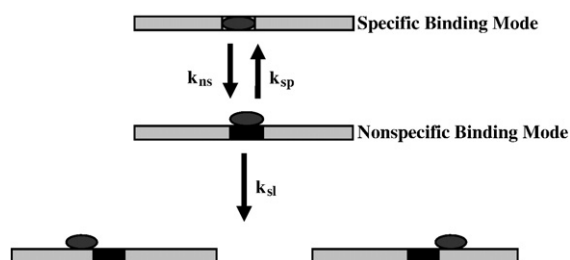


Fig. 4. A schematic representation of the kinetic steps involved in the transition from a specifically bound protein to a nonspecific complex abutting the specific site.

cleotide/fragment concentration ratios between ~ 100 and 1000. The reaction was stopped at various times by adding 10 μ l of triethylene glycol, as described by Rau.²⁷

Gel electrophoresis and quantitation

Samples were loaded onto a 12% polyacrylamide gel in 1 $\times$ Tris–acetate–EDTA buffer and electrophoresed at 180 V for ~ 14 h. The gel was stained with Sybr Green I (Invitrogen) and imaged with an FLA3000 (Fujifilm Life Sciences) laser scanner. The intensities of free DNA and protein–DNA complex bands were analyzed using Multi-gauge (Fujifilm Life Sciences) and SigmaPlot 10 (Systat Software).

Three bands are seen in the electrophoresis of the two-site fragment with EcoRI (Fig. 2a): free DNA, the complex with one bound protein, and, in the uppermost band, the complex with two bound proteins. The initial fractions of free DNA and complexes with one and two bound proteins were consistent with a simple binomial distribution of independent binding sites. The dissociation of the complex with two bound proteins to form a complex with one bound protein complicates the analysis of the real dissociation rate of the complex with one bound protein. The dissociation rate of the complex with two bound proteins can be measured from the loss of intensity of the upper band. If the fraction of DNA present as the complex with one protein is f_{1b} , the initial fraction of the complex with two bound proteins is f_{2b}^0 , and the rate constants for the loss of complexes with one and two bound proteins are k_1 and k_2 , respectively, then the fraction of DNA with one bound protein correcting for the contribution of the complex with two bound proteins $f_{1b,corr}$ is:

$$f_{1b,corr}(t) = f_{1b}(t) - \frac{k_2 f_{2b}^0}{k_1 - k_2} (e^{-k_2 t} - e^{-k_1 t}) \quad (9)$$

Iteration with the new k_1 changes the correction negligibly. The ratio of the dissociation rate of the one-site complex to the dissociation rate of the two-site fragment with two bound proteins varied between 1 and 1.8, suggesting that a bound protein is a reflecting barrier, as concluded by Jeltsch *et al.*¹¹

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2009.10.049](https://doi.org/10.1016/j.jmb.2009.10.049)

References

- Berg, O. G., Winter, R. B. & von Hippel, P. H. (1981). Diffusion-driven mechanisms of protein translocation on nucleic acids: models and theory. *Biochemistry*, **20**, 6929–6948.
- Gorman, J. & Greene, E. C. (2008). Visualizing one-dimensional diffusion of proteins along DNA. *Nat. Struct. Mol. Biol.* **15**, 768–774.
- Halford, S. E. & Marko, J. F. (2004). How do site-specific DNA-binding proteins find their targets? *Nucleic Acids Res.* **32**, 3040–3052.
- Winter, R. B., Berg, O. G. & von Hippel, P. H. (1981). Diffusion-driven mechanisms of protein translocation on nucleic acids: 3. The *Escherichia coli* lac repressor–operator interaction: kinetic measurements and conclusions. *Biochemistry*, **20**, 6961–6977.
- Winter, R. B. & von Hippel, P. H. (1981). Diffusion-driven mechanisms of protein translocation on nucleic acids: 2. The *Escherichia coli* repressor–operator interaction: equilibrium measurements. *Biochemistry*, **20**, 6948–6960.
- Berg, O. G. & von Hippel, P. H. (1985). Diffusion-controlled macromolecular interactions. *Annu. Rev. Biophys. Chem.* **14**, 131–160.
- Coppey, M., Benichou, O., Voituriez, R. & Moreau, M. (2004). Kinetics of target site localization of a protein on DNA: a stochastic approach. *Biophys. J.* **87**, 1640–1649.
- Cherstvy, A. G., Kolomeisky, A. B. & Kornyshev, A. A. (2008). Protein–DNA interactions: reaching and recognizing the targets. *J. Phys. Chem. B*, **112**, 4741–4750.
- Slutsky, M. & Mirny, L. A. (2004). Kinetics of protein–DNA interaction: facilitated target location in sequence-dependent potential. *Biophys. J.* **87**, 4021–4035.
- Halford, S. E. (2009). An end to 40 years of mistakes in DNA–protein association kinetics. *Biochem. Soc. Trans.* **37**, 343–348.
- Jeltsch, A., Alves, J., Wolfes, H., Maass, G. & Pingoud, A. (1994). Pausing of the restriction endonuclease EcoRI during linear diffusion on DNA. *Biochemistry*, **33**, 10215–10219.
- Jack, W. E., Terry, B. J. & Modrich, P. (1982). Involvement of outside DNA sequences in the major kinetic path by which EcoRI endonuclease locates and leaves its recognition sequence. *Proc. Natl Acad. Sci. USA*, **79**, 4010–4014.
- Jeltsch, A. & Pingoud, A. (1998). Kinetic characterization of linear diffusion of the restriction endonuclease EcoRV on DNA. *Biochemistry*, **37**, 2160–2169.
- Ehbrecht, H. J., Pingoud, A., Urbanke, C., Maass, G. & Gualerzi, C. (1985). Linear diffusion of restriction endonucleases on DNA. *J. Biol. Chem.* **260**, 6160–6166.
- Bonnet, I., Biebricher, A., Porte, P. -L., Loverdo, C., Benichou, O., Voituriez, R. *et al.* (2008). Sliding and jumping of single EcoRV restriction enzymes on non-cognate DNA. *Nucleic Acids Res.* **36**, 4118–4127.
- Laurence, T. A., Kwon, Y., Johnson, A., Hollars, C. W., O'Donnell, M., Camarero, J. A. & Barsky, D. (2008). Motion of a DNA sliding clamp observed by single molecule fluorescence spectroscopy. *J. Biol. Chem.* **283**, 22895–22906.
- Wang, Y. M., Cox, E. C. & Austin, R. (2006). Single molecule measurements of repressor protein 1D diffusion on DNA. *Phys. Rev. Lett.* **97**, 048302.
- Gorman, J., Chowdhury, A., Surtees, J. A., Shimada, J., Reichman, D. R., Alani, E. & Greene, E. C. (2007). Dynamic basis for one-dimensional DNA scanning by the mismatch repair complex Msh2–Msh6. *Mol. Cell*, **28**, 359–370.
- Blainey, P. C., van Oijen, A. M., Banerjee, A., Verdine, G. L. & Xie, X. S. (2006). A base-excision DNA-repair protein finds intrahelical lesion bases by fast sliding in contact with DNA. *Proc. Natl Acad. Sci. USA*, **103**, 5752–5757.
- Tafvizi, A., Huang, F., Leith, J. S., Fersht, A. R., Mirny, L. A. & van Oijen, A. M. (2008). Tumor suppressor p53 slides on DNA with low friction and high stability. *Biophys. J.* **95**, L01–L03.
- Komazin-Meredith, G., Mirchev, R., Golan, D. E., van Oijen, A. M. & Coen, D. M. (2008). Hopping of a processivity factor on DNA revealed by single molecule assays of diffusion. *Proc. Natl Acad. Sci. USA*, **105**, 10721–10726.

22. Viadiu, H. & Aggarwal, A. K. (2000). Structure of BamHI bound to nonspecific DNA: a model for sliding. *Mol. Cell*, **5**, 889–895.
23. Kalodimos, C. G., Biris, N., Bonvin, A. M. J. J., Levandoski, M. M., Guennuegues, M., Boelens, R. & Kaptein, R. (2004). Structure and flexibility adaptation in nonspecific and specific protein–DNA complexes. *Science*, **305**, 386–389.
24. Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P. *et al.* (1993). The crystal structure of EcoRV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* **12**, 1781–1795.
25. Sidorova, N. Y. & Rau, D. C. (1996). Differences in water release for the binding of EcoRI to specific and nonspecific DNA sequences. *Proc. Natl Acad. Sci. USA*, **93**, 12272–12277.
26. Sidorova, N. Y., Muradymov, S. & Rau, D. C. (2006). Differences in hydration coupled to specific and nonspecific competitive binding and to specific DNA binding of the restriction endonuclease BamHI. *J. Biol. Chem.* **281**, 35656–35666.
27. Rau, D. C. (2006). Sequestered water and binding energy are coupled in complexes of lambda Cro repressor with non-consensus binding sequences. *J. Mol. Biol.* **361**, 352–361.
28. Garner, M. M. & Rau, D. C. (1995). Water release associated with specific binding of gal repressor. *EMBO J.* **14**, 1257–1263.
29. Halford, S. E. (2001). Hopping, jumping and looping by restriction enzymes. *Biochem. Soc. Trans.* **29**, 824–834.
30. Iwahara, J., Zweckstetter, M. & Clore, G. M. (2006). NMR structural and kinetic characterization of a homeodomain diffusing and hopping on nonspecific DNA. *Proc. Natl Acad. Sci. USA*, **103**, 15062–15067.
31. Sidorova, N. Y. & Rau, D. C. (2004). Differences between EcoRI nonspecific and ‘star’ sequence complexes revealed by osmotic stress. *Biophys. J.* **87**, 2564–2576.
32. Belotserkovskii, B. P. & Zarling, D. A. (2004). Analysis of a one-dimensional random walk with irreversible losses at each step: applications for protein movement on DNA. *J. Theor. Biol.* **226**, 195–203.
33. Sidorova, N. Y. & Rau, D. C. (2001). Linkage of EcoRI dissociation from its specific DNA recognition site to water activity, salt concentration, and pH: separating their roles in specific and non-specific binding. *J. Mol. Biol.* **310**, 801–816.
34. Parsegian, V. A., Rand, R. P. & Rau, D. C. (2000). Osmotic stress, crowding, preferential hydration, and binding: a comparison of perspectives. *Proc. Natl Acad. Sci. USA*, **97**, 3987–3992.
35. Lesser, D. R., Kurpiewski, M. R. & Jen-Jacobson, L. (1990). The energetic basis of specificity in the EcoRI endonuclease–DNA interaction. *Science*, **250**, 776–786.
36. Schurr, J. M. (1978). The one-dimensional diffusion coefficient of proteins absorbed on DNA. Hydrodynamic considerations. *Biophys. Chem.* **9**, 413–414.
37. Bagchi, B., Blaney, P. C. & Xie, X. S. (2008). Diffusion constant of a nonspecifically bound protein undergoing curvilinear motion along DNA. *J. Phys. Chem. B*, **112**, 6282–6284.