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Solution parameters modulating DNA binding specificity of the restriction endonuclease EcoRV

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Summary

The DNA binding stringency of restriction endonucleases is crucial for their proper function. The x-ray structures of the specific and non-cognate complexes of the restriction nuclease EcoRV are considerably different suggesting significant differences in the hydration and binding free energies. Nonetheless, the majority of studies performed at pH 7.5, optimal for enzymatic activity, have found less than a 10-fold difference between EcoRV binding constants to the specific and nonspecific sequences in the absence of divalent ions. We used a recently developed self-cleavage assay to measure EcoRV-DNA competitive binding and to evaluate the influence of water activity, pH and salt concentration on the binding stringency of the enzyme in the absence of divalent ions. We find the enzyme can readily distinguish specific and nonspecific sequences. The relative specific-nonspecific binding constant increases strongly with increasing neutral solute concentration and with decreasing pH. The difference in number of associated waters between specific and nonspecific DNA-EcoRV complexes is consistent with the differences in the crystal structures. In spite of the large pH dependence of the sequence specificity, the osmotic pressure dependence indicates little change in structure with pH. The large osmotic pressure dependence means that measurement of protein-DNA specificity in dilute solution cannot be directly applied to binding in the crowded environment of the cell. In addition to divalent ions, water activity and pH are key parameters that strongly modulate binding specificity of EcoRV.

Keywords

DNA-protein specific binding; restriction endonucleases; equilibrium competition; water activity; gel electrophoresis

Introduction

Type II restriction endonucleases are paradigms of specificity for their ability to cleave recognition sequences while leaving nonspecific DNA intact in spite of its vast abundance over the specific site. All restriction endonucleases require divalent cations for cleavage, but they can vary in their ability to bind DNA specifically in the absence of divalent ions. A classical example of a protein with extreme binding specificity is the restriction endonuclease EcoRI that binds to its canonical site, GAATTC, with a constant $\sim 10^{11} \text{ M}^{-1}$ in 0.1 M salt in the absence of divalent ions. When any of the 6 bp is changed, binding affinity decreases by a factor 10^3 to 10^4 [1–3]. Yet another type II restriction endonuclease, EcoRV, requires divalent cations to achieve the same level of sequence selectivity as EcoRI. There

are, however, conflicting results in the literature regarding the ability of EcoRV restriction endonuclease to bind DNA specifically in the absence of divalent ions particularly at pH ~ 7.5 that is optimal for the EcoRV enzymatic activity. In their earlier studies, Taylor et al. [4], Thielking et al [5], Vermote et al. [6], Vipond and Halford [7], Alves et al. [8], Szczelkun and Connolly [9] employing the gel mobility shift assay concluded that EcoRV does not show any DNA sequence binding specificity in the absence of divalent ions. In contrast, Engler et al. [10] reported significant level of specificity for the binding of wild type EcoRV to the specific recognition sequence over nonspecific DNA sequences in the absence of divalent ions using both the gel mobility shift and filter binding assays. The ratio of specific and nonspecific binding constant was estimated about 155 at pH 7.4. Engler et al. [10] contended that if the gel running buffer had a pH > 7 (pH 8 – 8.3 was used by the other authors), then the gel retardation assay significantly underestimates the association binding constant. Later, Martin et al. [11] also using the gel mobility shift assay with a pH 7 running buffer disputed the results of Engler et al. [10] and reported that EcoRV binds to its specific sequence only 5-fold better than to a nonspecific site in the absence of divalent ions at pH 7.5 and ~ 10,000-fold better in the presence of Ca^{2+} . Reid et al. [12] measuring fluorescence anisotropy found that the preference of EcoRV for the specific sequence did not exceed ~ 6.5 fold in the absence of divalent ions at pH 7.5. Using fluorescence resonance energy transfer (FRET) and fluorescence anisotropy, Erskine and Halford [13] reported no difference between the equilibrium binding constants of EcoRV to specific and nonspecific sequences in the absence of divalent ions at pH 7.5.

The x-ray structures of the specific and noncognate complexes of EcoRV [14–15] in the absence of divalent cations are significantly different. The specific complex has mostly direct DNA-protein contacts at the interface and the DNA is highly bent; while the nonspecific complex has a large gap at the interface that is presumably water filled and the DNA is straight. This is similar to the difference between the specific and nonspecific complexes of BamHI with DNA [16–17]. Based on x-ray data alone it would be unexpected and counterintuitive that EcoRV-DNA specific and non-cognate complexes that have such different structures should have similar binding free energies [18]. In our experience, differences in the interface hydration of the DNA-protein complexes correlate with differences in binding free energies [2, 19–22]. However, the structures of the specific and nonspecific EcoRV-DNA complexes in solution may not be the same as seen by x-ray crystallography due to lattice interactions and packing energies. Indeed, Hiller et al [23] report that in solution DNA bending in the complex with EcoRV is only observed at pH 7.5 in the presence of divalent metal ions. This could indicate that the complex with the specific sequence in the absence of divalent cations resembles the non-cognate complex structurally. A lack of sequence specificity at pH 7.5 is then a natural consequence. Spectroscopic differences between the specific and nonspecific complexes in solution at pH 7.5, however, have been reported by Thorogood et al [24] and by Erskine and Halford [13]. As techniques based on separation, the gel mobility shift and filter binding assays have been criticized since the equilibrium distribution of free and protein-bound DNA could be disturbed during the experiment, and that could result in either under- or over-estimation of binding constants. In this study, we employ another technique developed by us previously. Using the observation that neutral solutes dramatically slow the dissociation of many DNA-protein specific complexes [19–20, 22, 25] we developed a self-cleavage solution assay [20, 26]. This assay uses the cleavage reaction of restriction endonucleases to measure sensitively their DNA binding. This technique does not have the limitations of the gel mobility shift or filter binding assays, but provides the same level of sensitivity. Additionally, contrary to other techniques, this method only measures enzymatically competent complexes that are capable of DNA cleavage in the presence of Mg^{2+} . Using this assay we measure the relative specific – nonspecific equilibrium binding constant through direct binding competition of the specific site with nonspecific sequences and its dependence on pH, salt concentration,

and osmotic pressure. Relative binding constants are not only straightforward to measure but are more directly relevant to binding specificity and dependence of specificity on different solution parameters. In agreement with Engler et al [10], we observe a strong pH dependence of the specific-nonspecific association binding constant ratio, increasing ~500-fold between pH 8.0 and 5.5. The sequence specificity of the EcoRV at pH 6.4 is comparable to the specificity of BamHI at pH 7.0. At pH 7.6, the ratio of association binding constants for a specific site 310 bp DNA fragment and a 30 bp nonspecific oligonucleotide, $K_{\text{nsp-sp}}$, is ~60 in the absence of divalent cations. This is indeed relatively low compared to both EcoRI and BamHI, but is still significantly larger than the 1–6.5-fold ratio reported previously.

We have also measured the osmotic pressure dependence of the specific-nonspecific competitive binding constant. This gives a measure of the difference between the two complexes in water associated with protein that is sequestered from osmolytes either sterically or by a preferential hydration, $\Delta N_{\text{w,nsp-sp}}$. We have found that specific, noncognate, and nonspecific DNA-protein complexes can be distinguished by differences in sequestered water [2, 20, 25]. Our previous results with EcoRI and BamHI showed more than 100 water molecules difference between the specific and nonspecific complexes [2], [20]. We concluded this water is in the cavity at the protein-DNA interface of the nonspecific complex, consistent with the insensitivity to osmolyte nature and with the x-ray structures for BamHI complexes. The binding specificity of EcoRV dramatically increases with increasing concentrations of neutral osmolytes, particularly triethylene glycol. The sensitivity to water activity for three of the four osmolytes used is consistent with the difference seen in the crystal structures without divalent cations. Even in the absence of divalent cations protein binds its specific DNA sequence in a specific-like mode. Contrary to both BamHI and EcoRI restriction endonucleases, $\Delta N_{\text{w,nsp-sp}}$ measured with triethylene glycol is significantly different from the other three osmolytes and would suggest there is a significant change in the exposed surface area between specific and noncognate DNA-EcoRV complexes in addition to the cavity at the interface of the noncognate complex. We see very little dependence of $\Delta N_{\text{w,nsp-sp}}$ on pH. In spite of the large change in $K_{\text{nsp-sp}}$ with pH, the structures of the specific and nonspecific complexes likely change minimally.

Results

Self-cleavage assay optimization for measuring EcoRV-DNA specific binding

The basis of the self-cleavage assay is that the distribution of enzyme-bound and free specific site DNA fragment is ‘trapped’ by adding a large concentration of osmolyte to greatly slow dissociation of the enzyme from the recognition site and competitor oligonucleotide also containing the specific recognition site to bind excess enzyme and to prevent rebinding to the DNA fragment. Mg^{2+} is added to allow the cleavage reaction to proceed. The cleavage reaction is stopped by adding EDTA. We will refer to the enzyme trapped on the specific site of the DNA fragment as enzymatically competent even though the fully active enzyme conformation that can actually cleave DNA may only evolve with added Mg^{2+} . The concentrations of both osmolyte and oligonucleotide are variables for optimization. Control experiments indicate that final reaction conditions of 20 mM imidazole pH 6.5–6.8, 100 mM NaCl, 10 mM MgCl_2 , 400-fold molar excess of specific site oligonucleotide over specific site fragment, and 3 osmolal triethylene glycol are sufficient for the efficient ‘trapping’ of the complex. A cleavage mix is added to the preformed complex to result in these solution conditions. There is less than 2% difference in the fraction of enzyme-bound fragment if Mg^{2+} is added immediately with the cleavage mix or 60 min after the rest of the cleavage mix (data not shown). The triethylene glycol effectively stops dissociation. Nor does it matter if complexes are incubated for 10 min or 30 min in cleavage mix with Mg^{2+} before adding EDTA. The 400-fold excess of specific site

oligonucleotide is sufficient to prevent rebinding of enzyme to DNA fragment (Figure S1 in Supplementary data). In all experiments described further in this work, DNA-protein samples were incubated with cleavage mix at 20 °C for 20 min.

Kinetics of EcoRV-DNA binding

The time needed to reach equilibrium depends sensitively on association and dissociation rates. Figure 1 shows the kinetics of DNA-protein complex formation measured by the self-cleavage assay for different experimental conditions of pH and osmotic pressure. Each time point corresponds to the incubation time of EcoRV (~ 1.5 nM) with specific site 310 bp DNA fragment (~ 3 nM) before self-cleavage mix is added. Virtually all protein was bound at equilibrium for the experiments shown. The final fraction of bound DNA at long times $f_{b,\infty}$, range from 0.52 to 0.58. The binding of EcoRV proceeds with at least two time constants. About 55% of the total protein binds to the DNA in an enzymatically competent conformation much faster than the minute time-scale of our experiment. It takes ~ 1.5–4 hours for the remaining 45% of the protein to form an enzymatically active complex with the DNA specific fragment. This unexpected result was also reproduced with commercial EcoRV from New England Biolabs (data not shown).

The time dependence of the slow component kinetics for complex formation can be well described by a single exponential. The rate constant of the slow component is significantly pH dependent. The half-life time of the slow component measured in the presence of 1 osmolal triethylene glycol increases from ~19 min. at pH 6.3 to ~42 min at pH 7.6. There was no measurable difference in the half-life time of the slow component measured at pH 7.6 in the presence of 1 or 2 osmolal triethylene glycol. Nor do we observe that a two-fold change in EcoRV concentration at pH 6.8 affects the kinetics of complex formation (Figure S2 in Supplementary data). We also performed a control experiment using the self-cleavage assay to measure the rate of EcoRI association to its specific sequence fragment with the same experimental conditions and protocol used for EcoRV. EcoRI was completely bound within 2 minutes (our fastest time point) of incubation of protein with DNA (Figure S3 in Supplementary data).

The slow kinetics of complex formation at pH 7.6 necessitates an incubation time of at least 4–5 hours to ensure that equilibrium is reached. The specific site complex is stable for at least 24 hours as determined by the self-cleavage assay. To avoid adjusting incubation times in the equilibrium competition experiments separately for each set of conditions, we chose to incubate DNA-EcoRV complexes for 18–20 hours before adding cleavage mix.

In contrast to association, the dissociation kinetics of the EcoRV can be well described by a single exponential (Fig. S4). The rates are sufficiently fast under all experimental conditions used in this study such that 18–20 hours incubation was enough to reach equilibrium (data not shown).

EcoRV-DNA specific binding measured with the gel mobility shift and self-cleavage assays

The electrophoretic mobility shift assay (EMSA) [27–28] is a widely used tool for quantitating DNA-protein binding. The technique requires that the complex is stable once in the gel and that the distribution of complex and free DNA remains unchanged in the electrophoretic well before entering the gel. Engler et al [10] has reported that the running buffer pH should be ~7.0, rather than the standard 8.3 with TAE or TBE, in order to stabilize the EcoRV-DNA complex. We observed similar problems at pH 8.3 compared with pH 7.0 and suspect that the dissociation rate at pH 8.3 is too fast for the EMSA. The diffusion and electrophoresis of protons is much faster than any other solution component, and samples are exposed to quickly changing conditions of pH while in the electrophoretic well [26]. We

have further modified the standard EMSA protocol in order to ensure that the distribution of complex and DNA fragment is stable by adding triethylene glycol to further slow dissociation and specific site oligonucleotide to prevent binding of free protein to the specific site DNA fragment [26], but no Mg^{2+} . Figure 2 shows a comparison of EcoRV binding measurement using the gel shift and self-cleavage assays. The gel mobility shift assay is shown on the left-hand side of Fig.2A and the self-cleavage assay on the right. The gel was run with a pH 6.9 running buffer (imidazole) using our protocol. For both techniques, the complex was incubated overnight under conditions of stoichiometric binding before assaying.

Figure 2B shows the analysis of the gels presented on Fig.2A. Both titration dependencies are linear as expected for virtually stoichiometric protein binding. The fractions of DNA bound measured by the self-cleavage and the gel mobility shift assays are practically indistinguishable. This result further confirms that both techniques give reliable and quantitative results under proper conditions.

The relative specific-nonspecific binding constant of EcoRV and its osmotic pressure dependence

The relative binding constant, K_{nsp-sp} , is the ratio of the association binding constants, K_{sp}/K_{nsp} , for EcoRV binding to a 310 bp specific site DNA fragment and a 30 bp nonspecific oligonucleotide and was measured from direct equilibrium competition experiments. Mixtures of EcoRV, the 310 bp specific sequence fragment, and varying concentrations of a nonspecific oligonucleotide competitor were incubated at 20 °C for 18–20 hours. The loss of the specific site binding as the concentration of competing nonspecific oligonucleotide increased was determined by the self-cleavage assay. Figure 3A shows a gel image illustrating the competition for EcoRV binding between the nonspecific oligonucleotide and the specific site DNA fragment for 0.4 and 0.8 osmolal triethylene glycol at pH 6.8 and 100 mM NaCl. Under these conditions EcoRV binds virtually stoichiometrically (< 5% free protein) to the 310 bp DNA fragment in the absence of oligonucleotide making calculation of K_{nsp-sp} quite straightforward. Figure 3B shows the analysis of the gel shown in Fig. 3A. The relative binding constant K_{nsp-sp} can be calculated from the slopes of the lines using Eq. 1 from the “Materials and methods”. Analogous experiments were performed for three other solutes.

Figure 4 shows the osmotic pressure dependence of $\ln(K_{nsp-sp})$ at pH 6.8 for the four osmolytes examined, triethylene glycol, betaine glycine, TMAO, and α -methyl glucoside. The sensitivity to osmotic pressure indicates a difference in the exclusion of osmolytes from the water associated with specific and nonspecific complexes. Slopes of the lines can be translated into a difference in the number of complex associated water molecules that are consequently included, $\Delta N_{w,sp-nsp}$, using equation (3) of Materials and methods. Since less water is sequestered by the specific complex as seen in the crystal structures, specific binding is strongly favored over nonspecific binding by the presence of neutral solutes. The osmotic dependence of the difference of binding free energy between specific and nonspecific binding (in units of kT) is linear for all four osmolytes indicating that $\Delta N_{w,sp-nsp}$ is constant for each solute over the range of osmotic pressures examined. $\Delta N_{w,sp-nsp}$ values are, however, dependent on the osmolyte used ranging from 114 ± 4 water molecules seen with betaine to 224 ± 14 water molecules using triethylene glycol. In contrast, $\Delta N_{w,sp-nsp}$ was virtually insensitive to the osmolyte identity for seven solutes used in analogous competition experiments for BamHI [20] and EcoRI [2] restriction endonucleases.

Figure 4 confirms that EcoRV is quite proficient at distinguishing between specific and nonspecific DNA sequences in the absence of divalent cofactor at pH 6.8. The average

competitive binding constant, $K_{\text{nsp-sp}}$, with no added osmolyte is ~ 274 . Impressively, in the presence of only 1 osmolal triethylene glycol this ratio increases 55-fold, to $\sim 15,000$.

The pH dependence of $K_{\text{nsp-sp}}$ for EcoRV-DNA binding

Figure 5 shows the dependence of the specific-nonspecific binding free energy difference on triethylene glycol osmolal concentration measured at pH 6.3, 6.8 and 7.6. All three curves are linear with slopes translating into $\Delta N_{\text{w,nsp-sp}}$ equal to 226 ± 5 at pH 7.6, 224 ± 14 at pH 6.8, and 281 ± 15 at pH 6.3. $K_{\text{nsp-sp}}$ measured in the absence of triethylene glycol, changes from 56 ± 6 at pH 7.6, to 283 ± 36 at pH 6.8 and to 1173 ± 154 at pH 6.3. We do see a strong increase of the relative binding constant with decreasing pH in agreement with results obtained earlier by Engler et al [10]. Nonetheless, even at pH 7.6, EcoRV is still able to distinguish between specific and nonspecific sequences on DNA in the absence of osmolytes. As a further confirmation of these results, specific site fragment complex was titrated with either specific site oligonucleotide or nonspecific oligonucleotide at pH 7.6 and 100 mM NaCl. This result is additionally illustrated on Figure S5 (Supplementary data). Less than 9% of the specific fragment-EcoRV complex formed in the absence of oligonucleotides is still present when 30-fold molar excess of specific site oligonucleotide over specific fragment is added, but more than 73% is stable at the same excess of the nonspecific oligonucleotide. In the presence of 1 osmolal triethylene glycol, $K_{\text{nsp-sp}}$ at pH 7.6 increases from ~ 56 to $\sim 3,000$. $K_{\text{nsp-sp}}$ values at $\Pi_{\text{osm}} = 0$ and $\Delta N_{\text{w,nsp-sp}}$ values measured at pH 6.3, 6.8, and 7.6 are given in Table 1 for the four osmolytes used. Control experiments showed that the relative binding constant for the competition between the specific DNA fragment and a 30 bp oligonucleotide containing the specific recognition site is nearly one for pH 6.3, 6.8, and 7.6 (data not shown).

Figure 6 shows a titration curve for the pH dependence of $K_{\text{nsp-sp}}$ at $\Pi_{\text{osm}} = 0$ for the range of pH values 5.5 to 8. $K_{\text{nsp-sp}}$ is almost 3×10^4 at pH 5.5. An apparent plateau value for $K_{\text{nsp-sp}}$ at ~ 60 is observed at the higher pH values, but no plateau was observed in the lower range.

The salt dependence of $K_{\text{nsp-sp}}$ for EcoRV-DNA binding

A sensitivity of $K_{\text{nsp-sp}}$ to pH would suggest that the dependence of $K_{\text{nsp-sp}}$ on salt concentration should also vary with pH. Figure 7 shows the salt dependence of the $K_{\text{nsp-sp}}$ measured for the range of salt concentrations 60 to 140 mM NaCl measured at pH 6.3 and 7.6. The linear dependence of $\log(K_{\text{nsp-sp}})$ on $\log[\text{NaCl}]$ can be translated into a difference in the number of thermodynamically bound sodium ions between the nonspecific and specific complexes. At pH 7.6, the competitive binding constant, $K_{\text{nsp-sp}}$, increases slightly with increasing salt concentration indicating that the specific complex binds 1.5 ± 0.1 more sodium ions than the nonspecific complex. At pH 6.3, $K_{\text{nsp-sp}}$ is negligibly dependent on salt concentration suggesting that formation of both the specific and nonspecific DNA-EcoRV complexes releases the same number of sodium ions.

Discussion

X-ray structures for specific and non-cognate DNA-EcoRV complexes solved in the absence of metal co-factors [14–15] are noticeably different suggesting that there should be significant differences in hydration and binding free energies between two complexes as has been seen for EcoRI and BamHI complexes with DNA [2, 20]. Nonetheless, the majority of biochemical studies performed over last 20 years show either very little difference in binding free energies between specific and nonspecific DNA-EcoRV complexes in the absence of divalent cations, or none at all [4, 6–9, 11–13, 23, 29]. These investigations were performed at $\sim$ pH 7.5, the optimal pH for enzymatic activity for EcoRV. One group only

[10] reported a significant EcoRV specificity in the absence of divalent cations, $K_{\text{nsp-sp}} \sim 155$ at pH 7.4 for the competition of ~ 20 bp specific and nonspecific oligonucleotides.

Here we have used a self-cleavage solution assay developed by us [26] to measure EcoRV binding. This assay monitors only enzymatically competent complexes. We showed that under proper conditions the self-cleavage and gel mobility assays give identical results. Equilibrium measurements require knowledge of association and dissociation rates. We found that, under the conditions used here, EcoRV has unusual kinetics of specific complex formation in the absence of divalent ions that was not observed for EcoRI. A significant fraction of the total enzyme, $\sim 45\%$, forms enzymatically competent complexes unusually slowly (Figure 1). Rates of complex formation are slowest in the pH range ($\sim$ pH 7.5) that is most controversial for enzyme specificity. It would be quite easy to underestimate the specific binding constant if reaction mixture was not incubated long enough. In the experiment on complex formation (illustrated on Figure 1, filled squares) binding at equilibrium is stoichiometric (more than 95% of the protein is in DNA-bound state). The minimal value for the equilibrium dissociation constant can be estimated as at least $\sim 11.3 \times 10^9 \text{ M}^{-1}$. In majority of studies, 30 min incubation was considered sufficient to reach equilibrium. If the value for the equilibrium constant was calculated from the fraction DNA bound after 30 min (Figure 1, filled squares) it would be estimated as only $1.12 \times 10^9 \text{ M}^{-1}$, at least 10-fold lower.

We do not know the reason for such slow kinetics. Heterogeneity of the enzyme population could potentially be an artifact of a given preparation, but we observe slow association kinetics with both EcoRV isolated by us and from New England Biolabs. Additionally, the slowly associating component is fully capable of cleaving DNA. Only a single component is apparent in the dissociation also using the self-cleavage assay. The association kinetics of EcoRI using the same self-cleavage protocol shows no such slow component. The slow component is not a consequence of the assay. Preliminary data indicates that the fraction of the slow component depends sensitively on solution conditions. The 0.45 fraction of slowly associating protein reflects its presence in our enzyme storage buffer (see Materials and methods). Other research groups have reported much faster rates [13, 23, 30–31], but there are significant differences between our measurements and previous studies on the EcoRV association kinetics that prevent direct comparison with previous data. The majority of studies were performed in the presence of divalent cations. We were specifically interested in the EcoRV binding equilibrium in the absence of divalent ions, so association kinetics was also measured in the absence of divalent ions. We do not know yet how divalent cations and temperature affect the equilibrium between kinetic components. A strong dependence of the association kinetics rate on divalent ions concentration was reported before for the restriction endonuclease PvuII [32–33] that shows low binding stringency in the absence of divalent ions similar to EcoRV [34]. Hiller et al. [23] measured association kinetics of the EcoRV both in the presence and in the absence of divalent metals and found the on-rate to be even faster in the absence of divalent co-factors. It is not clear, however, if the plateau fluorescence anisotropy observed corresponds to complete enzyme binding. A slowly associating component could have been missed.

The rate of complex formation we observe for the EcoRV is not sensitive to protein concentration measured over a two-fold change or to the presence of the strongly excluded osmolyte triethylene glycol, suggesting that protein-protein interactions are not responsible for the two kinetic components, but that two conformations of the protein are present in solution. The x-ray structure of the free enzyme [14] shows that the DNA enveloping arms of the EcoRV are in a ‘closed’ conformation. Erskine et al. [35] and Schulze et al. [36] suggested that free EcoRV may exist in ‘closed’ and ‘opened’ conformations in solution; the existence of ‘opened’ and ‘closed’ conformations of another restriction endonuclease,

BsoBI, in the solution was recently demonstrated [37]. Work is currently in progress to further characterize the slowly associating component, the equilibrium distribution between slowly and fast associating forms of protein, and their exchange kinetics. The purpose of the kinetics experiment for this study was to determine incubation times necessary to establish EcoRV equilibrium binding.

We measured the ratio of association binding constants of EcoRV to a 310 bp DNA fragment containing the specific recognition site, K_{sp} , and a 30 bp nonspecific oligonucleotide, K_{nsp} , using the self-cleavage assay and varying osmotic pressure, pH, and salt. The strong pH dependence of the relative binding constant is in qualitative agreement with the results of Engler et al. [10]. A significant pH dependence of binding specificity was observed also for another type II restriction endonuclease, MunI [38]. Although both K_{nsp} and K_{sp} increase significantly with decreasing pH, we previously observed no pH dependence of K_{nsp-sp} for EcoRI [25]. Only a weak pH dependence for specific and nonspecific binding of PvuII, a close relative of EcoRV was seen both in the absence and presence of divalent metal ions [34].

At the lower pH values (<6.5), K_{nsp-sp} for EcoRV is comparable to the competitive binding constants at pH 7.0 for EcoRI ($\sim 1-2 \times 10^4$) [2, 25] and BamHI ($\sim 2 \times 10^3$) [20]. At pH 7.6 that maximizes enzyme activity, binding specificity is surprisingly low compared with EcoRI and BamHI. Even so it is still significantly higher than has been reported elsewhere. If we assume that EcoRV spans $\sim 10-15$ bp [14], then the ratio of association binding constants for binding to the recognition sequence and to a single $10-15$ bp nonspecific site is $\sim 800 - 1100$, the product of K_{nsp-sp} and the number of possible nonspecific sites on the 30 bp nonspecific oligonucleotide. This is then quite specific. The factor of ~ 60 difference (measured at pH 7.6) between binding to the 310 bp specific site DNA fragment that has ~ 300 nonspecific sites and to a nonspecific 30 bp oligonucleotide that contains some 20 possible nonspecific sites would also suggest that the specific site DNA fragment should have a significant fraction of nonspecifically bound protein, $\sim 30\%$ of the total protein bound to the specific site. The fraction of nonspecifically bound protein would be negligible though ($\sim 1\%$ of the total protein bound to the specific site) at pH 6.3 where K_{nsp-sp} is ~ 1200 . Nonetheless, the ratio of equilibrium constants for binding to the 310 bp specific site DNA fragment and to a specific site 30 bp oligonucleotide remains the same in the limit of experimental error at both pH 6.3 and 7.6. The self-cleavage assay protocol does not stabilize EcoRV nonspecifically bound to the DNA fragment long enough to find the recognition site and register as specifically bound.

A pH dependence of K_{nsp-sp} would indicate a difference in DNA-protein charge interactions between the specific and nonspecific complexes that should be consequently linked to a difference in salt concentration sensitivity. Figure 7 shows that between pH 7.6 and 6.3, the specific complex binds ~ 1.5 more ions than the nonspecific complex.

The osmotic pressure dependence of K_{nsp-sp} reports on the difference between specific and nonspecific complexes in the number of water molecules associated with complex that exclude osmolyte, $\Delta N_{w,nsp-sp}$. Osmolytes can be excluded from water associated with DNA-protein complexes due to either a steric exclusion from cavities or a preferential hydration of exposed protein and DNA surfaces ([39] and references cited there). An exclusion of solutes necessarily means an inclusion of water. As with BamHI [16–17], a major structural difference is the presence of a gap between the DNA and EcoRV protein interfaces in the nonspecific complex that is not present in the specific complex that has mainly direct protein-DNA contacts [14–15]. Once osmolytes are sufficiently large that they are sterically excluded from this cavity, the contribution from this gap to $\Delta N_{w,nsp-sp}$ will not depend on the solute nature. The size of this cavity for the EcoRV nonspecific complex is comparable

to that seen for BamHI [17]. The expected contribution to $\Delta N_{w, \text{nsp-sp}}$ from the difference between the DNA-protein interfaces of the specific and nonspecific complexes is $\sim 100\text{--}150$ water molecules/complex. The difference in the number of included water molecules between the specific and nonspecific complexes due to a preferential hydration will depend on both the natures of the osmolyte and of the protein and DNA surfaces and on the change in exposed surface area between the two structures. The $\Delta N_{w, \text{nsp-sp}}$ values for betaine glycine, α -methyl glucoside, and TMAO are reasonably consistent, 115–150 waters, suggesting a dominating contribution from the cavity for these osmolytes compared with a difference in exposed surface area. More osmolyte variation is observed for EcoRV, however, than we previously reported for EcoRI and BamHI [2, 20]. The observed $\Delta N_{w, \text{nsp-sp}}$ for triethylene glycol, ~ 224 at pH 6.8 (Figure 6), is quite different from the other solutes and would indicate a significant difference in exposed surface area between the specific and nonspecific complexes of EcoRV in addition to the cavity. We have found that triethylene glycol is particularly effective in stabilizing specific complexes through exclusion from exposed surfaces compared with α -methyl glucoside and betaine glycine [20–22, 25]. The large osmotic pressure dependence of $K_{\text{nsp-sp}}$ observed for EcoRV is comparable with that seen for EcoRI and BamHI that have much larger sequence specificities in the absence of divalent cations. Even though Hiller et al. [23] did not observe a DNA bend in the specific complex without divalent cations, the protein and DNA still seem to make the direct, specific complex-like contacts that are necessary to account for the large difference in sequestered water between complexes with specific and nonspecific sequences. The large osmotic pressure dependence observed for $K_{\text{nsp-sp}}$ also means that measurement of protein-DNA specificity in dilute solution cannot be directly applied to binding in the crowded environment of the cell. Osmotic pressure is a thermodynamic parameter that is as important as salt concentration and pH.

The strong pH dependence of $K_{\text{nsp-sp}}$ (Figure 6 and Table 1) in the absence of divalent ions might suggest that the structures of the specific or nonspecific EcoRV complexes are pH dependent. The insensitivity of $\Delta N_{w, \text{nsp-sp}}$ for betaine glycine to pH in the range 6.3 to 7.6, however, would suggest that the cavity at the protein-DNA of the nonspecific complex and the more direct association of the recognition DNA and protein surfaces of the specific complex remain unchanged with pH to within ~ 10 water molecules. The enzyme is binding DNA in a specific manner with direct DNA-protein contacts even at pH 7.6. The observation of a full water complement at pH 7.6 implies that $K_{\text{nsp-sp}}$ cannot be small. If there was no difference between EcoRV binding to nonspecific and specific sequences at pH 7.6, then only the nonspecific mode of binding would be realized on the specific sequence and $\Delta N_{w, \text{nsp-sp}}$ would be zero. If the specific and nonspecific binding modes of the EcoRV on the recognition site had the same binding free energy, then both structures would be equally probable at the recognition site and $\Delta N_{w, \text{nsp-sp}}$ would be half that for the actual difference between specific and nonspecific complexes, not the full value measured (Figure 5 and Table 1). The more substantial increase in $\Delta N_{w, \text{nsp-sp}}$ for triethylene glycol from 225 to 284 ($\sim 25\%$) as the pH is lowered from 7.6 to 6.3 would suggest a further change in exposed surface area of either the specific or nonspecific complex. Major structural changes in either the specific or the nonspecific complex, however, do not seem to occur over the pH range examined.

Several experiments shown in Figure 5 were done under conditions such that protein binding was not virtually stoichiometric. We can estimate the equilibrium dissociation binding constant of the specific EcoRV - DNA complex at pH 7.6, 100 mM NaCl and no osmolyte as $\sim 2\text{--}4$ nM. This value is in a reasonably good agreement with value of ~ 3 nM reported by Engler et al for pH 7.4 and 105 mM NaCl. For each pH we can also determine the minimal osmolyte concentration at which EcoRV specific binding in the absence of nonspecific competitor oligonucleotide becomes practically stoichiometric (defined as $>95\%$ protein

binding to DNA). For all three pH values, specific sequence stoichiometric binding is reached when $K_{\text{nsp-sp}} \sim 1,200$ implying that K_{sp} changes with pH and that K_{nsp} is relatively pH insensitive. This is consistent with the conclusions of Engler et al [10]. Since K_{nsp} seems relatively insensitive to pH, we conclude that the specific complex releases some 1.5 additional ions at pH 6.3 compared with 7.6. We cannot find titratable histidine groups that are in close contact with DNA in the specific complex but not in the nonspecific complex structure. We, therefore, agree with several groups [11–12, 18, 38] that the negatively charged amino acids in the active site of the enzyme are responsible for the pH dependence of $K_{\text{nsp-sp}}$ and of K_{sp} . Binding divalent ions to these sites would neutralize the excess negative charge at pH 7.6. $K_{\text{nsp-sp}}$ with added divalent would then more closely approximate $K_{\text{nsp-sp}}$ at much lower pH values without divalent cations.

Conclusions

We have re-examined the specificity of EcoRV restriction endonuclease binding using a self-cleavage assay that only monitors the formation of enzymatically competent complexes. There are several binding properties of this enzyme that distinguish it from both EcoRI and BamHI restriction endonucleases.

The binding specificity of the EcoRV is strongly pH dependent (again quite contrary to the EcoRI). The salt dependence of $K_{\text{nsp-sp}}$ is also pH dependent suggesting that differences in DNA-protein charge-charge interactions between the specific and nonspecific complex accompany pH changes.

The difference between the binding free energies of specific and nonspecific complexes strongly depends on neutral solute concentration. The osmotic pressure dependence of $K_{\text{nsp-sp}}$ for three of the four osmolytes examined is consistent with a dominating contribution from the cavity at the protein-DNA interface seen in the x-ray structure of the nonspecific complex. Contrary to both EcoRI and BamHI, however, $\Delta N_{\text{w,nsp-sp}}$ depends on the nature of the osmolyte used to set the osmotic pressure; triethylene glycol in particular is highly excluded from the specific complex compared with the nonspecific one. This solute sensitivity suggests that differences between specific and nonspecific complexes are not limited by the cavity seen at the DNA-protein interface in the nonspecific complex. There is an additional conformational change in surface exposed area between two structures. In spite of the large changes in $K_{\text{nsp-sp}}$ with pH, $\Delta N_{\text{w,nsp-sp}}$ measured using betaine glycine has very little pH sensitivity suggesting that the structures of the specific and nonspecific complexes change minimally with pH. The pH dependence of $\Delta N_{\text{w,nsp-sp}}$ measured using triethylene glycol would suggest there is a slight change in exposed surface area.

Lastly, EcoRV restriction endonuclease binds DNA specifically in the absence of divalent cations. Even at pH 7.6, the binding constant to the single specific site is about 1000-fold higher than binding constant to a single nonspecific site. In addition to divalent metal ions, water activity, and pH are key parameters that strongly modulate binding specificity of the EcoRV.

Materials and methods

Materials

A 533 bp DNA fragment containing a single EcoRV recognition sequence was isolated from the SphI and HindIII digestion of pBR322 plasmid using standard techniques. A 310 bp long DNA fragment containing one EcoRV cognate sequence was then obtained by PCR of the 533 bp fragment using internal primer sequences. The 24 nt PCR primer oligonucleotides used to generate the 310 bp fragment were complementary to bp # 80–104 and 366–390 of

pBR322; the EcoRV site is at bp #185. Cleavage of the 310 bp DNA fragment with the EcoRV produces DNA fragments of 107 bp and 203 bp long. The pBR322 plasmid and restriction enzymes SphI and HindIII were purchased from New England Biolabs. The primer oligonucleotides were purchased from Invitrogen.

The sequences of the double-stranded 30 bp long EcoRV specific site and nonspecific oligonucleotides used were: 5'-CGGGCCTCTTGCGGGATATCGTCCATTCCG-3' and 5'-CGGGCCTCTTGCGGCTATAGGTCCATTCCG-3' respectively. The EcoRV recognition sequence is shown in bold. The EcoRV nonspecific sequence oligonucleotide has the same sequence except that the recognition sequence is replaced with an inverted EcoRV recognition sequence (underlined). The double stranded oligonucleotides were prepared as described in [20]. The specific sequence double-stranded oligonucleotide was additionally purified on a Protein Pak anion exchange column (Waters) using AKTA Purifier (GE Healthcare Life Sciences). Double-stranded oligonucleotides were then ethanol precipitated, and dissolved in TE buffer (10 mM TrisCl (pH 7.5), 1 mM EDTA). The purity of the double-stranded oligonucleotides was confirmed by polyacrylamide gel electrophoresis. The concentrations of the DNA fragment and double-stranded oligonucleotides were determined spectrophotometrically, using an extinction coefficient of $0.013 \text{ (cm} \cdot \mu\text{M base-pairs)}^{-1}$ at 260 nm.

DNA binding and cleavage experiments were performed with highly purified EcoRV restriction endonuclease (described below) or with commercial EcoRV sample purchased from New England Biolabs. Active protein concentrations of the EcoRV were determined by the direct titration with the specific site 310 bp DNA fragment under conditions of stoichiometric binding as described previously [2, 20].

Betaine glycine, α -methyl glucoside, triethylene glycol, and trimethylamine N-oxide (TMAO) were purchased from Fluka Analytical. All solutes were used without further purification. Osmolal concentrations of solutes were determined by direct measurement using a vapor pressure osmometer operating at room temperature (Wescor, model 5520XR).

EcoRV Purification

EcoRV was purified using a modified procedure of the method developed by Luke et al. [40]. *Escherichia coli* strain CSH50 [pMetB] as well as the plasmid mix pBSKSRVD/pMetB were kind gifts of Dr. A. Prota and Dr. F. Winkler. Plasmid pMetB (constructed to overproduce EcoRV methylase) is described in [29]. pBSKSRVD plasmid is a modification [41] of the original plasmid constructed for EcoRV nuclease overproduction [42]. Cell disruption step was performed using CellLytic protocol/reagents from Sigma Chemical. Column chromatography was performed using an AKTA Purifier system (GE Healthcare Life Sciences). The protein was initially bound to a phosphocellulose column in 20 mM K_2HPO_4 (pH 7.0), 1 mM EDTA, 10 mM DTT, 10% glycerol, and 0.2 M NaCl. EcoRV eluted at ~ 0.4 M NaCl using a salt gradient. The protein was further purified using a HiTrap Blue Sepharose column (GE Healthcare Life Sciences). The enzyme was eluted with a linear gradient of 0.2 to 0.8 M NaCl also in 20 mM K_2HPO_4 (pH 7.0), 1 mM EDTA, 10 mM DTT, and 10% glycerol and came off the column at 0.675 M NaCl. Purity of the EcoRV endonuclease was confirmed by SDS-polyacrylamide gel electrophoresis. The purified protein was aliquoted and stored at -80°C . Enzyme stock solutions routinely used in the experiments were adjusted to 10 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 5 mM DTT, 338 mM NaCl, 55% glycerol and 200 $\mu\text{g/ml}$ acetylated BSA and were stored at -20°C . We determine active protein concentrations by titration to the specific DNA fragment under conditions of stoichiometric binding as described in [20]. There is no measurable decrease in protein activity after 18 months storage at -20°C .

Self-cleavage assay

To measure the fraction of DNA with specifically bound EcoRV by the self-cleavage assay, described in detail in [20, 26], 30 μ l of a cleavage mixture containing imidazole buffer, triethylene glycol, MgCl_2 , and specific site oligonucleotide was added to pre-equilibrated 30 μ l samples of EcoRV-DNA complexes. The composition of the cleavage mixture was adjusted to ensure a final pH of 6.5–6.8 and final concentrations of 10 mM MgCl_2 , 100 mM NaCl, and 400-fold molar excess of specific site oligonucleotide competitor over the specific site fragment. Enough triethylene glycol was added to the cleavage mixture to ensure a final total osmotic pressure of 3 osmolal. Samples were then incubated for 20 min. at 20 °C; the cleavage reaction was stopped by adding EDTA to a final concentration of 20 mM. DNA digestion products were purified using GenElute PCR Clean-up kit (Sigma Chemical Co., Inc.).

Equilibrium binding experiments

Binding reaction mixtures contained 20 mM imidazole (for the pH range 6.3 – 8.0) or 20 mM MES buffer (for the pH range 5.7 – 6.7), 60 – 140 mM NaCl, 2 mM DTT, 50 μ g/ml acetylated BSA and 0.1 mM EDTA. The total reaction volume was 30 μ l. Specific-nonspecific equilibrium competition experiments were performed as described previously [2, 20]. Briefly, mixtures of EcoRV (~1.5 nM), the specific site fragment (~3 nM), and the nonspecific oligonucleotide competitor (between 0 and ~50 μ M in oligonucleotide), were incubated at 20 °C overnight to ensure equilibrium. The loss of specific site binding as the concentration of nonspecific competitor DNA increased was determined by the self-cleavage assay.

Association and dissociation kinetics

Solution conditions for the EcoRV kinetic experiments were 20 mM imidazole (pH 6.3, 6.8, and 7.6), 100 mM NaCl, 2 mM DTT, 50 μ g/ml BSA, and 0.1 mM EDTA. To measure complex formation kinetics EcoRV (~1.5 nM) was incubated with the 310 bp specific site DNA fragment (~3 nM). At timed intervals 30 μ l aliquots were withdrawn from the DNA-protein pool and fractions of specifically bound EcoRV assayed using the self-cleavage technique.

Dissociation kinetics was measured using a standard approach. EcoRV (~1.5 nM) was initially incubated at 20 °C overnight with the 310 bp specific site DNA fragment (~3 nM). EcoRV specific sequence oligonucleotide was then added to a final 400–4000 fold excess molar concentration over DNA fragment. The fraction of specifically bound EcoRV was then determined at timed intervals using the self-cleavage assay. In this case, the cleavage mixture contained only Mg^{2+} and triethylene glycol. Specific site oligonucleotide was omitted from cleavage mixture since significant excess of the oligonucleotide was already present in the reaction mixture.

Gel electrophoresis

Ficoll was added to the DNA digestion products from the self-cleavage assay to a final concentration of 3% and samples were then loaded on a 9% polyacrylamide gel, the running buffer was TAE (22.5 mM Tris, 11.25 mM acetic acid, 0.5 mM EDTA, pH 8.3). Samples were run at 350 V for 2.5–3 hours.

We have modified the standard protocol for gel mobility shift experiments in order to prevent perturbation of the equilibrium between free DNA and complex in the electrophoretic well. A stop reaction mixture (30 μ l) was added to each 30 μ l sample to 'trap' the equilibrium fraction of specifically bound DNA [26]. The composition of the stop reaction mixture was such to ensure that the final mix contained ~400-fold molar excess of

the specific site oligonucleotide over the specific site fragment and enough triethylene glycol to ensure a final osmotic pressure of 3 osmolal. Samples were then loaded on 9% polyacrylamide gel; the running buffer was 20 mM imidazole (pH 6.9). Samples were electrophoresed at 350 V for 3.5–4 hours. Electrophoretic bands containing free DNA, and DNA-protein complex (in case of the gel mobility shift assay), or uncleaved and cleaved DNA fragments (self-cleavage assay) were stained with the fluorescent dye SYBR Green I (Molecular Probes). The gels were imaged with a FLA-3000 Fluorescent Image Analyzer (Fuji Film). The FLA-3000 was interfaced to a Pentium PC. Band intensities were quantified using the Fuji Film software MultiGauge for Windows.

Equilibrium competition analysis

As was developed previously [2, 20], the ratio of specific (sp) and nonspecific (nsp) association binding constants (K_{sp}/K_{nsp}) can be determined from the loss of specific complex as the concentration of a nonspecific oligonucleotide competitor is increased. If f_b and f_b^0 are the fractions of protein-bound specific site fragment with and without added oligonucleotide competitor, respectively, then under conditions of virtually stoichiometric protein binding ($<5\%$ free protein) and for much weaker nonspecific than specific binding ($K_{nsp} \ll K_{sp}$), the concentration of nonspecific complex can be easily calculated as equal to $(f_b^0 - f_b) \cdot [DNA_{sp}]_{total}$. Specific sequence binding is given then by,

$$f_b = f_b^0 - \frac{K_{nsp}}{K_{sp}} \frac{f_b}{1 - f_b} \frac{[DNA_{nsp}]_{total}}{[DNA_{sp}]_{total}}, \quad \text{Eq. 1}$$

where $[DNA_{sp}]_{total}$ is the molar concentration of specific sequence fragment, and $[DNA_{nsp}]_{total}$ is the molar concentration of nonspecific oligonucleotide. Competitive binding constants, K_{sp}/K_{nsp} , were straightforwardly calculated from the linear dependence of fraction bound DNA, f_b , on $f_b [DNA_{nsp}]_{total} / ((1 - f_b) [DNA_{sp}]_{total})$, measured at constant specific sequence DNA and protein concentrations. We also performed equilibrium competition experiments under nonstoichiometric binding conditions. In this case, the concentration of nonspecific complex, $[DNA_{nsp}]_{bound}$, cannot be calculated directly from the loss of specific binding. The concentrations of free protein and of the nonspecific complex should be calculated using the specific association constant, K_{sp} that can be determined from binding in the absence of competitor (described in details in [20]). The relative specific-nonspecific binding constant can then be straightforwardly calculated,

$$K_{nsp-sp} = \frac{K_{sp}}{K_{nsp}} = \frac{f_b [DNA_{nsp}]_{total}}{(1 - f_b) [DNA_{nsp}]_{bound}}. \quad \text{Eq. 2}$$

All values of K_{nsp-sp} given here are the average of 3–4 experiments.

The difference in the numbers of solute excluding water molecules between specifically and nonspecifically bound protein can be calculated from the dependence of K_{sp}/K_{nsp} on the solute osmolal concentration [2] by

$$\frac{d \ln(K_{nsp-sp})}{d[\text{osmolal}]} = \frac{d \ln(K_{sp}/K_{nsp})}{d[\text{osmolal}]} = - \frac{\Delta N_{w,nsp-sp}}{55.6}, \quad \text{Eq. 3}$$

where $\Delta N_{w,ns\text{-}sp} = N_{w,sp} - N_{w,ns\text{-}sp}$, the difference in the numbers of water molecules associated with specific and nonspecific complexes that exclude solute.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lesser DR, Kurpiewski MR, Jen-Jacobson L. The energetic basis of specificity in the Eco RI endonuclease-DNA interaction. *Science*. 1990; 250:776–786. [PubMed: 2237428]
2. Sidorova NY, Rau DC. Differences in water release for the binding of EcoRI to specific and nonspecific DNA sequences. *Proc Natl Acad Sci U S A*. 1996; 93:12272–12277. [PubMed: 8901570]
3. Thielking V, Alves J, Fliess A, Maass G, Pingoud A. Accuracy of the EcoRI restriction endonuclease: binding and cleavage studies with oligodeoxynucleotide substrates containing degenerate recognition sequences. *Biochemistry*. 1990; 29:4682–4691. [PubMed: 2372551]
4. Taylor JD, Badcoe IG, Clarke AR, Halford SE. EcoRV restriction endonuclease binds all DNA sequences with equal affinity. *Biochemistry*. 1991; 30:8743–8753. [PubMed: 1909572]
5. Thielking V, Selent U, Kohler E, Landgraf A, Wolfes H, Alves J, Pingoud A. Mg²⁺ confers DNA binding specificity to the EcoRV restriction endonuclease. *Biochemistry*. 1992; 31:3727–3732. [PubMed: 1567826]
6. Vermote CL, Halford SE. EcoRV restriction endonuclease: communication between catalytic metal ions and DNA recognition. *Biochemistry*. 1992; 31:6082–6089. [PubMed: 1627551]
7. Vipond IB, Halford SE. Specific DNA recognition by EcoRV restriction endonuclease induced by calcium ions. *Biochemistry*. 1995; 34:1113–1119. [PubMed: 7827059]
8. Alves J, Selent U, Wolfes H. Accuracy of the EcoRV restriction endonuclease: binding and cleavage studies with oligodeoxynucleotide substrates containing degenerate recognition sequences. *Biochemistry*. 1995; 34:11191–11197. [PubMed: 7669777]
9. Szczelkun MD, Connolly BA. Sequence-specific binding of DNA by the EcoRV restriction and modification enzymes with nucleic acid and cofactor analogues. *Biochemistry*. 1995; 34:10724–10733. [PubMed: 7662656]
10. Engler LE, Welch KK, Jen-Jacobson L. Specific binding by EcoRV endonuclease to its DNA recognition site GATATC. *J Mol Biol*. 1997; 269:82–101. [PubMed: 9193002]
11. Martin AM, Horton NC, Lusetti S, Reich NO, Perona JJ. Divalent metal dependence of site-specific DNA binding by EcoRV endonuclease. *Biochemistry*. 1999; 38:8430–8439. [PubMed: 10387089]
12. Reid SL, Parry D, Liu HH, Connolly BA. Binding and recognition of GATATC target sequences by the EcoRV restriction endonuclease: a study using fluorescent oligonucleotides and fluorescence polarization. *Biochemistry*. 2001; 40:2484–2494. [PubMed: 11327870]
13. Erskine SG, Halford SE. Reactions of the eco RV restriction endonuclease with fluorescent oligodeoxynucleotides: identical equilibrium constants for binding to specific and non-specific DNA. *J Mol Biol*. 1998; 275:759–772. [PubMed: 9480767]
14. Winkler FK, Banner DW, Oefner C, Tsernoglou D, Brown RS, Heathman SP, Bryan RK, Martin PD, Petratos K, Wilson KS. The crystal structure of EcoRV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *Embo J*. 1993; 12:1781–1795. [PubMed: 8491171]

15. Horton NC, Perona JJ. Role of protein-induced bending in the specificity of DNA recognition: crystal structure of EcoRV endonuclease complexed with d(AAAGAT) + d(ATCTT). *J Mol Biol.* 1998; 277:779–787. [PubMed: 9545372]
16. Newman M, Strzelecka T, Dorner LF, Schildkraut I, Aggarwal AK. Structure of Bam HI endonuclease bound to DNA: partial folding and unfolding on DNA binding. *Science.* 1995; 269:656–663. [PubMed: 7624794]
17. Viadiu H, Aggarwal AK. Structure of BamHI bound to nonspecific DNA: a model for DNA sliding. *Mol Cell.* 2000; 5:889–895. [PubMed: 10882125]
18. Winkler, FK.; Protta, AE. Structure and Function of EcoRV Endonuclease. In: Pingoud, A., editor. *Restriction Endonucleases.* Springer-Verlag; Berlin: 2003. p. 179-214.
19. Sidorova NY, Rau DC. Differences between EcoRI nonspecific and “star” sequence complexes revealed by osmotic stress. *Biophys J.* 2004; 87:2564–2576. [PubMed: 15454451]
20. Sidorova NY, Muradymov S, Rau DC. Differences in hydration coupled to specific and nonspecific competitive binding and to specific DNA Binding of the restriction endonuclease BamHI. *J Biol Chem.* 2006; 281:35656–35666. [PubMed: 17008319]
21. Garner MM, Rau DC. Water release associated with specific binding of gal repressor. *Embo J.* 1995; 14:1257–1263. [PubMed: 7720716]
22. Rau DC. Sequestered water and binding energy are coupled in complexes of lambda Cro repressor with non-consensus binding sequences. *J Mol Biol.* 2006; 361:352–361. [PubMed: 16828799]
23. Hiller DA, Fogg JM, Martin AM, Beechem JM, Reich NO, Perona JJ. Simultaneous DNA binding and bending by EcoRV endonuclease observed by real-time fluorescence. *Biochemistry.* 2003; 42:14375–14385. [PubMed: 14661948]
24. Thorogood H, Waters TR, Parker AW, Wharton CW, Connolly BA. Resonance Raman spectroscopy of 4-thiothymidine and oligodeoxynucleotides containing this base both free in solution and bound to the restriction endonuclease EcoRV. *Biochemistry.* 1996; 35:8723–8733. [PubMed: 8679635]
25. Sidorova NY, Rau DC. 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.* 2001; 310:801–816. [PubMed: 11453689]
26. Sidorova NY, Muradymov S, Rau DC. Trapping DNA-protein binding reactions with neutral osmolytes for the analysis by gel mobility shift and self-cleavage assays. *Nucleic Acids Res.* 2005; 33:5145–5155. [PubMed: 16155185]
27. Fried M, Crothers DM. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 1981; 9:6505–6525. [PubMed: 6275366]
28. Garner MM, Revzin A. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic Acids Res.* 1981; 9:3047–3060. [PubMed: 6269071]
29. Vermote CL, Vipond IB, Halford SE. EcoRV restriction endonuclease: communication between DNA recognition and catalysis. *Biochemistry.* 1992; 31:6089–6097. [PubMed: 1627552]
30. Sam MD, Perona JJ. Mn²⁺-dependent Catalysis by Restriction Enzymes: Pre-Steady-State Analysis of EcoRV Endonuclease Reveals Burst Kinetics and the Origins of Reduced Activity. *J Am Chem Soc.* 1999; 121:1444–1447.
31. Hancock SP, Hiller DA, Perona JJ, Jen-Jacobson L. The energetic contribution of induced electrostatic asymmetry to DNA bending by a site-specific protein. *J Mol Biol.* 2011; 406:285–312. [PubMed: 21167173]
32. Xie F, Qureshi SH, Papadakis GA, Dupureur CM. One- and two-metal ion catalysis: global single-turnover kinetic analysis of the PvuII endonuclease mechanism. *Biochemistry.* 2008; 47:12540–12550. [PubMed: 18975919]
33. Conlan LH, Dupureur CM. Multiple metal ions drive DNA association by PvuII endonuclease. *Biochemistry.* 2002; 41:14848–14855. [PubMed: 12475233]
34. Conlan LH, Dupureur CM. Dissecting the metal ion dependence of DNA binding by PvuII endonuclease. *Biochemistry.* 2002; 41:1335–1342. [PubMed: 11802735]
35. Erskine SG, Baldwin GS, Halford SE. Rapid-reaction analysis of plasmid DNA cleavage by the EcoRV restriction endonuclease. *Biochemistry.* 1997; 36:7567–7576. [PubMed: 9200708]

36. Schulze C, Jeltsch A, Franke I, Urbanke C, Pingoud A. Crosslinking the EcoRV restriction endonuclease across the DNA-binding site reveals transient intermediates and conformational changes of the enzyme during DNA binding and catalytic turnover. *Embo J.* 1998; 17:6757–6766. [PubMed: 9822618]
37. Dikic, J. The conformational dynamics of BsoBI, analyzed by fluorescence spectroscopy down to the single molecule level. Justus-Liebig-University; Giessen: 2009.
38. Haq I, O'Brien R, Lagunavicius A, Siksnyš V, Ladbury JE. Specific DNA recognition by the type II restriction endonuclease MunI: the effect of pH. *Biochemistry.* 2001; 40:14960–14967. [PubMed: 11732916]
39. Parsegian VA, Rand RP, Rau DC. Osmotic stress, crowding, preferential hydration, and binding: A comparison of perspectives. *Proc Natl Acad Sci U S A.* 2000; 97:3987–3992. [PubMed: 10760270]
40. Luke PA, McCallum SA, Halford SE. The EcoR V restriction endonuclease. *Gene Amplif Anal.* 1987; 5:185–207. [PubMed: 3333365]
41. Vipond IB, Halford SE. Random mutagenesis targeted to the active site of the EcoRV restriction endonuclease. *Biochemistry.* 1996; 35:1701–1711. [PubMed: 8639649]
42. Bougueleret L, Tenchini ML, Botterman J, Zabeau M. Overproduction of the EcoR V endonuclease and methylase. *Nucleic Acids Res.* 1985; 13:3823–3839. [PubMed: 2989776]

Fig. 1.

Kinetics of the EcoRV-DNA complex formation. The kinetics of DNA-protein complex formation was measured using the self-cleavage assay at different conditions of pH: pH 6.3 (▲); pH 7.6 (■). The binding of the EcoRV proceeds in at least two steps. About 60% of the protein binds within the first 5 minutes of the kinetic experiment. The time dependence of the remaining slow component can be well described by the single exponential (fits are shown for both curves). The fraction of bound (cleaved) DNA was normalized by the limiting plateau value, $f_{b,\infty}$, for each curve. The rate of the slow component is significantly pH dependent. The half-life time of the slow component measured in the presence of 1 osmolal triethylene glycol increases from ~19 min. at pH 6.3 to ~42 min. at pH 7.6. EcoRV and DNA were initially incubated in 20 mM imidazole (pH 6.3 or 7.6), 100 mM NaCl and 1 osmolal triethylene glycol for the indicated periods of time before assaying.

Fig. 2.

A direct comparison of EcoRV-DNA binding analyzed by the gel mobility shift assay and by the self-cleavage assay. (A) A gel image is shown illustrating a direct comparison of the EcoRV-DNA binding by the gel mobility shift assay (left), and by the self-cleavage assay (right). Stop reaction mixture to stabilize the complex/free DNA fragment distribution in the electrophoretic well was added to the gel mobility shift samples (up to 400-fold excess specific site oligonucleotide and 3 osmolal triethylene glycol in the final sample). Cleavage mixture (up to 10 mM MgCl₂, 400-fold excess molar specific site oligonucleotide, and 3

osmolal triethylene glycol in the final sample) was used in the self-cleavage assay. **(B)** The calculated fraction of total DNA fragment with bound protein as dependent on the total protein added is shown for the gels in **(A)**. Both the gel mobility shift (●) and the self-cleavage assay (Δ) give practically identical measures of EcoRV binding. For both techniques, complexes were incubated at 20 °C overnight in 20 mM imidazole (pH 6.8), 100 mM NaCl, and 1 osmolal triethylene glycol before assaying.

Fig. 3.

Equilibrium competition between specific and nonspecific DNA sequences for the EcoRV binding. Mixtures of EcoRV, the 310 bp DNA fragment with a specific recognition site, and nonspecific oligonucleotide competitor were incubated at 20 °C overnight in the presence of 0.4 or 0.8 osmolal triethylene glycol, 20 mM imidazole (pH 6.8), and 100 mM NaCl. **(A)** The loss of specific site binding as the concentration of nonspecific competitor increased was determined by the self-cleavage assay. Only DNA fragments with initially bound enzyme are cleaved. Less cleavage is observed as the nonspecific oligonucleotide concentration is increased, indicating a loss of specific binding. **(B)** The ratio of the association binding constants for the specific site DNA fragment and the nonspecific

oligonucleotide, $K_{\text{nsp-sp}}$, is extracted from the loss of specific binding as the concentration of nonspecific oligonucleotide increases. The fraction of protein-bound DNA fragment, f_b , is plotted against the parameter $f_b[\text{DNA}_{\text{nsp}}]/((1-f_b)[\text{DNA}]_{\text{sp}})$ as given by Eq. 1 in “Materials and methods” for the case of stoichiometrically bound protein. The slope of the best fitting straight line is $-1/K_{\text{nsp-sp}}$. For 0.4 osmolal triethylene glycol (●), $K_{\text{nsp-sp}}=1411\pm123$; for 0.8 osmolal triethylene glycol (▲), $K_{\text{nsp-sp}}=8606\pm290$.

Fig. 4.

The dependence of the EcoRV specific-nonspecific binding free energy difference, $\ln(K_{\text{nsp-sp}})$ in the units of kT , on solute osmolal concentration is shown for four neutral osmolytes. Mixtures of the specific site DNA fragment, nonspecific oligonucleotide, and EcoRV were prepared at 100 mM NaCl, 20 mM imidazole pH 6.8, and different concentrations of neutral solutes. Mixtures were incubated at 20 °C overnight. Competitive binding constants for betaine glycine (●), α -methyl glucoside (Δ), trimethylamine N-oxide (TMAO) (◆), and triethylene glycol ($\square$) were measured using the self-cleavage assay as described in “Materials and methods”. Changes in competitive binding free energies scale linearly with osmolal concentration or, equivalently, with water chemical potential for all solutes shown. The difference in solute-excluded water molecules, $\Delta N_{\text{w,nsp-sp}}$, between specific and nonspecific complexes, can be calculated for each solute from linear fits to the data using Eq. 3 in “Materials and methods”. The best fitting lines give $\Delta N_{\text{w,nsp-sp}}$ equal to 114 ± 4 waters for betaine glycine; 127 ± 2 waters for methyl glucoside; 150 ± 10 waters for TMAO; 224 ± 14 waters for triethylene glycol. Error bars for most points are on the order of the size of symbols.

Fig. 5.

The dependence of the EcoRV specific-nonspecific binding free energy difference on triethylene glycol concentration is shown for different pH values. Mixtures of EcoRV, the 310 bp specific site DNA fragment, and the nonspecific oligonucleotide competitor were incubated at 20 °C overnight in the presence of different concentrations of triethylene glycol in 100 mM NaCl and 20 mM imidazole (pH 6.3 (▲), pH 6.8 (□), and pH 7.6 (●)). The fraction of DNA bound to EcoRV was measured using the self-cleavage assay. Changes in competitive binding free energies scale linearly with triethylene glycol osmolal concentration for each pH value shown. The best fitting lines (Eq. 3 in “Materials and methods”) give $\Delta N_{w,nsp-sp}$ values of 281 ± 15 at pH 6.3, 224 ± 14 at pH 6.8, and 226 ± 5 at pH 7.6.

Fig. 6.

pH dependence of the EcoRV specific-nonspecific free binding energy difference. The pH dependence of $\ln(K_{\text{nsp-sp}})$ is shown for the range 5.5 to 8.0. Mixtures of EcoRV, the 310 bp specific site DNA fragment, and the nonspecific oligonucleotide competitor were incubated at 20 °C overnight in the absence of osmolytes in 100 mM NaCl and either in 20 mM MES buffer (Δ), or in 20 mM imidazole buffer ($\bullet$). The competitive binding constant, $K_{\text{nsp-sp}}$, at each pH was measured using the self-cleavage assay. An apparent plateau value for $K_{\text{nsp-sp}}$ at ~ 60 was observed at higher pH values, but no plateau was observed in the lower range.

Fig. 7.

Salt dependence of the EcoRV specific-nonspecific free binding energy difference measured at pH 6.3 and 7.6. The salt dependencies of $\log(K_{\text{nsp-sp}})$ measured for the range of salt concentrations 60 to 140 mM NaCl at either pH 6.3 (▲) or at pH 7.6 (■) are shown. Mixtures of EcoRV, the 310 bp specific site DNA fragment, and the nonspecific oligonucleotide competitor were incubated overnight at 20 °C in the absence of osmolytes in 20 mM imidazole at different NaCl concentrations. The competitive binding constant, $K_{\text{nsp-sp}}$, at each salt concentration was measured using the self-cleavage assay. The linear dependence of $\log(K_{\text{nsp-sp}})$ on $\log([NaCl])$ can be translated into a difference in the number of thermodynamically bound sodium ions between the nonspecific and specific complexes. At pH 7.6, the specific complex binds 1.5 ± 0.1 more sodium ions than the nonspecific complex. At pH 6.3, $K_{\text{nsp-sp}}$ is negligibly dependent on salt concentration with the slope translated into only about -0.35 ± 0.3 sodium ions.

The ratio between specific and nonspecific EcoRV binding constants measured at the conditions of no osmolyte ($K^0_{\text{nsp-sp}}$) and corresponding difference in number of water molecules ($\Delta N_{\text{w,nsp-sp}}$) released for the binding of EcoRV to specific and to nonspecific DNA sequences are shown for four osmolytes at different pH values. Values for $\Delta N_{\text{w,nsp-sp}}$ and $K^0_{\text{nsp-sp}}$ were determined from linear fits of data as shown in Fig. 4 and 5.

Table 1

Osmolyte	pH 6.3		pH 6.8		pH 7.6	
	$K^0_{\text{nsp-sp}}$	$\Delta N_{\text{w,nsp-sp}}$	$K^0_{\text{nsp-sp}}$	$\Delta N_{\text{w,nsp-sp}}$	$K^0_{\text{nsp-sp}}$	$\Delta N_{\text{w,nsp-sp}}$
Betaine	1250±133	117±7	249±22	114±4	56±10	126±9
α -Methyl glucoside	-	-	281±5	127±2	64±15	142±11
TMAO	-	-	283±53	150±10	-	-
Triethylene glycol	1173±154	281±15	285±50	224±14	56±6	226±5