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Using Single-Turnover Kinetics with Osmotic Stress to Characterize the EcoRV Cleavage Reaction

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Abstract

Type II restriction endonucleases require metal ions to specifically cleave DNA at canonical sites. Despite the wealth of structural and biochemical information, the number of Mg^{2+} ions used for cleavage by EcoRV, in particular, at physiological divalent ion concentrations is still not established. In this work we employ a single-turnover technique that uses osmotic stress in order to probe reaction kinetics between an initial specific EcoRV-DNA complex formed in the absence of Mg^{2+} and the final cleavage step. With osmotic stress, complex dissociation before cleavage is minimized and the reaction rates are slowed to a convenient timescale of minutes to hours. We find that cleavage occurs by a two-step mechanism that can be characterized by two rate constants. The dependence of these rate constants on Mg^{2+} concentration and osmotic pressure gives the number of Mg^{2+} ions and water molecules coupled to each kinetic step of the EcoRV cleavage reaction. Each kinetic step is coupled to the binding 1.5 – 2.5 Mg^{2+} ions, the uptake of ~30 water molecules, and the cleavage of a DNA single strand. We suggest that each kinetic step reflects an independent, rate limiting conformational change of each monomer of the dimeric enzyme that allows Mg^{2+} ion binding. This modified single turnover protocol has general applicability for metalloenzymes.

Metal ions play an important role in biology, serving as essential co-factors for a wide variety of cellular enzymes. In particular, divalent metal ions play a central role in nuclease cleavage reactions (1–4). In the presence of Mg^{2+}, type II restriction endonucleases function as precise molecular scissors, cleaving unmethylated DNA at the canonical sites with exquisite specificity thereby protecting bacteria from foreign DNA invasion. Despite the wealth of structural and biochemical information available on the role of metal ions in the cleavage reactions (reviewed in (1–4)), the exact number of Mg^{2+} required for cleavage is still unclear. It has been difficult to determine unequivocally the number of divalent ions needed for cleavage experimentally. The number and identity of metal ions required for DNA cleavage by nucleases are most commonly inferred from x-ray structures. Somewhat unexpectedly, structurally similar restriction nucleases from the EcoRI family have been crystallized with different numbers of metal ions (5). Divalent metal ion binding to three distinct sites per monomer have been seen in different crystals of EcoRV complexes (6–9) but are never all occupied by divalent metal ions at the same time. Horton and Perona (7) have attempted to reconcile the three-metal binding sites suggesting that only two Mg^{2+} ions per monomer are necessary for cleavage proposing that the two Mg^{2+} ions shift among the

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Supporting Information Available

The predicted dependence of rate constants on Mg^{2+} concentration is shown assuming ♠ N = 1, 2, and 3, demonstrating that the data well discriminates among these values. Secondly, without osmotic stress, dissociation of specifically bound EcoRV is faster than Mg^{2+} binding and DNA cleavage for standard conditions of pH 7.5 and 100 mM NaCl. This material is available free of charge via the Internet at http://pubs.acs.org.

three sites during the cleavage reaction. Surprisingly, if crystals of the EcoRV- specific DNA complex formed in the absence of Mg^{2+} are then equilibrated against a solution containing 30 mM Mg^{2+}, the resulting crystals had two Mg^{2+} ions bound to one monomer of the dimer and none to the other (8). This Mg^{2+} binding did not induce DNA cleavage. Only one of the Mg^{2+} binding sites diffracted strongly; the other binding site was only weakly occupied with Mg^{2+}. The strongly diffracting Mg^{2+} is in a position to catalyze DNA phosphate hydrolysis and is considered the primary Mg^{2+}. The more weakly diffracting Mg^{2+} seems to be auxiliary and is thought to increase the cleavage rate by the primary Mg^{2+}. The crystal structure of EcoRV with product DNA, i.e., cleaved oligonucleotide, showed two Mg^{2+} ions bound per monomer at the primary and auxiliary sites (8).

Biochemical experiments performed by different research groups have found between one and two Mg^{2+} ions per EcoRV monomer as critical for cleavage. Typically, both Mg^{2+} and Mn^{2+} can catalyze cleavage reactions of restriction nucleases; whereas the enzyme is inactive with only Ca^{2+} present. The most straightforward demonstration that two metal ions per monomer are needed for cleavage comes from the observation that several restriction nucleases are more active with both Mg^{2+} or Mn^{2+} and Ca^{2+} added rather than with only Mg^{2+} or Mn^{2+} (5, 10). At fixed Mn^{2+} or Mg^{2+} concentration, Vipond et al. (10) found that k_{cat} of EcoRV varied linearly with Ca^{2+} concentration indicating that only one Ca^{2+} binds per dimer during the cleavage step, suggesting that at least three divalent ions per dimeric enzyme are sufficient for cleavage, the two Mn^{2+} or Mg^{2+} ions bound at the primary sites and the Ca^{2+} that is presumed to bind more tightly to the auxillary site.

Xie et al (11) globally fit a large set of binding and cleavage kinetics for the type II restriction endonuclease PvuII, which shares many common properties with the EcoRV. The best fit indicated that the enzyme can cleave DNA when only one metal ion occupies the active site of a monomer, but that the cleavage rate is ~100-fold slower than if two metal ions are bound per monomer. The authors concluded for PvuII and possibly other restriction enzymes of the same family that only one metal ion is strictly necessary for cleavage with second ion modulating the activity. This is supported by studies with EcoRV mutated at Glu45 that is part of the presumed secondary Mg^{2+} binding site. The E45A mutant nuclease still has cleavage activity, but at a reduced level from the wild type (12). In contrast, mutations of Asp90 and Asp74 that comprise the primary divalent ion binding site completely abolish activity (12). Molecular dynamics simulations (5) have indicated that one Mg^{2+} at the primary site is sufficient for cleavage for restriction nucleases in the EcoRI family. In contrast, however, the simulations of Zahran et al (13) indicated that each monomer of the EcoRV requires two magnesium ions for cleavage. Zahran et al predicted that in the absence of the second ion, the protein-DNA hydrogen bond network would be significantly disrupted and that the sharp kink at the central base pair of the recognition site would essentially disappear.

The dependence of kinetic rates or equilibrium binding constants on salt concentration or pH is routinely used to determine the numbers of ions or protons coupled to the reaction. The Mg^{2+} concentration dependence of EcoRV cleavage rates indicated that two divalent ions per dimer are linked to the reaction (12). Single-turnover kinetic experiments eliminate dissociation kinetics from traditional cleavage rate experiments. In traditional single-turnover kinetic experiments, a large excess of enzyme is added to DNA ensuring pseudo first order kinetics and that only one reaction round of DNA cleavage occurs. The stopped flow kinetic experiments of Baldwin et al (14) are most relevant to the work presented here. Tryptophan fluorescence was used to monitor EcoRV conformational changes. Adding Mg^{2+} to EcoRV already bound to DNA showed cleavage kinetics that had a lag phase and that could be fit to two exponentials. The assumption was that the enzyme did not dissociate before cleaving the DNA; thus eliminating the enzyme-DNA binding step from the reaction

scheme. The Mg^{2+} dependence of the second step that results in double-strand cleavage indicated that only one Mg^{2+} binds per dimer in the concentration range 0.25 – 6 mM. The Mg^{2+} dependence of the first step associated with the lag phase was more complicated. For Mg^{2+} concentrations less than ~ 1 mM, Mg^{2+} was actually released. From ~1 to 4 mM Mg^{2+}, no Mg^{2+} dependence of the rate constant is observed. For larger Mg^{2+} concentrations, extra Mg^{2+} is bound, but the amount is difficult to quantitate.

We have devised our own single-turnover experimental protocol to measure the numbers of Mg^{2+} ions, H^+, and water coupled to the cleavage kinetics of EcoRV. Despite the traditional view that the restriction endonuclease EcoRV is not capable of specific site recognition in the absence of divalent ions at pH ~7.5, we recently reported (15) that this enzyme is in fact capable of binding preferentially to its cognate sequence on DNA in the absence of divalent co-factors. We also found that in addition to metal co-factors, osmotic pressure and pH are two key parameters that strongly modulate the enzyme binding strength and specificity. At pH 7.6 and 100 mM NaCl the ratio between EcoRV binding constants to the specific 310 bp long DNA fragment and to nonspecific 30 bp long oligonucleotide is only ~ 56, but this ratio increases to ~ 2.4×10^3 in the presence of 1.7 osmolal betaine glycine. The specific sequence binding constant, K_a, increases from ~ 2×10^8 M^{-1} to ~ 2×10^{10} M^{-1} as the betaine glycine concentration is increased from 0 to 1.7 osmolal for 100 mM NaCl and pH 7.6. At pH 6.3 the ratio between specific and nonspecific binding constants is ~ 1.2×10^3 in the absence of solute; in the presence of 1.7 osmolal concentration of glycine betaine it increases to ~4.5×10^4. This large increase is because the specific EcoRV-DNA complex sequesters some 120 fewer water molecules from glycine betaine than the nonspecific complex. Other solutes, triethylene glycol, TMAO, and methyl glucoside influence the EcoRV binding specificity in a similar way (15) confirming that solutes act osmotically, i.e. by changing water activity. This sensitivity to osmotic pressure is a common feature of many protein-DNA complexes (15–27). Since the specific to nonspecific mode of protein binding is a necessary initial step in dissociation of the specific complex, osmotic pressure also greatly slows the dissociation rate. We utilize osmotic stress in our single-turnover experimental protocol to ensure that the cleavage reaction is initiated with virtually all protein bound to the specific DNA fragment and that dissociation of the complex before cleavage is minimized. We additionally prevent rebinding of any dissociated enzyme by including high concentrations of competitor oligonucleotide to trap free enzyme. In this way we specifically measure the numbers of Mg^{2+}, H^+, and water molecules coupled to the cleavage kinetics between the initial specific EcoRV-DNA complex with no bound Mg^{2+} and the transition cleavage state. This is an extension of the self-cleavage assay we developed previously to measure specific binding of the restriction endonucleases through their cleavage with high precision (15, 27, 28). The foundation for this technique is that specifically bound enzyme in the absence of Mg^{2+} can be stoichiometrically converted to the active form simply by adding Mg^{2+}. An additional feature of using osmotic pressure in the single-turnover kinetic experiments is that since the kinetic steps leading to cleavage require water uptake, the kinetics are slowed to a time scale of minutes to hours, allowing convenient measurement.

We find that the EcoRV cleavage kinetics can be well described by two consecutive reaction steps. Each kinetic step is remarkably similar, coupled to the binding of 1.5–2.4 Mg^{2+} ions, the release of 2 H^+ ions in the pH range 6.0 to 6.8, the binding of ~30 water molecules, and the cleavage of a single DNA strand. The simplest explanation is that each monomer of EcoRV undergoes a conformational change associated with Mg^{2+} binding. To the best of our knowledge, this is the first direct thermodynamic determination of the number of Mg^{2+} needed for cleavage by EcoRV and was only possible using osmotic stress.

Materials and methods

Materials

The 310 bp DNA fragment containing the EcoRV recognition sequence was isolated as described in (28). Briefly, the 533 bp DNA fragment containing a single EcoRV recognition sequence was isolated from the SphI and HindIII digestion of pBR322 plasmid using standard techniques. The 310 bp fragment was then obtained from the 533 bp fragment using PCR with internal primers. Cleavage of the 310 bp fragment at the EcoRV recognition site yields DNA fragments of 107 bp and 203 bp long. pBR322 plasmid and restriction enzymes were ordered from New England Biolabs. The primer oligonucleotides were purchased from Invitrogen. The supercoiled plasmid Litmus28i was purchased from New England Biolabs and used without further purification.

The sequence of the double-stranded 30 bp long EcoRV specific site oligonucleotide used to trap free enzyme was: 5′-CGGGCCTCTTGCGG**GATATC**GTCCATTCCG-3′ (the EcoRV recognition sequence is shown in bold). The specific site oligonucleotide and its complement were purchased from Invitrogen and dissolved in STE buffer (100 mM NaCl, 10 mM Tris-Cl (pH 7.5), and 1 mM EDTA). Double-stranded 30 bp oligonucleotides containing the EcoRV specific cleavage site were prepared as described in (15, 27). After annealing and purification using P6 Bio-Spin columns double-stranded oligonucleotides were ethanol precipitated and dissolved in TE buffer (10 mM TrisCl (pH 7.5), 1 mM EDTA). The purity was checked by polyacrylamide gel electrophoresis. The concentrations of the DNA fragment and double-stranded oligonucleotides were determined spectrophotometrically, using an extinction coefficient of 0.013 $(cm \cdot mM\ base\text{-}pairs)^{-1}$ at 260 nm. Absorption spectra were obtained with a Perkin Elmer Lambda 800 UV-VIS spectrophotometer.

Glycine betaine (purchased from Fluka/Sigma) osmolal concentrations were measured using a vapor pressure osmometer at room temperature (Wescor, model 5520XR).

DNA cleavage experiments were performed with highly purified EcoRV restriction endonuclease (described below).

EcoRV purification

The EcoRV purification procedure was a modified form of the method developed by Luke et al. (29) and previously described in (15). Enzyme stock solutions used for experiments contained 10 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 5 mM DTT, 338 mM NaCl, 55% glycerol, 200 μg/ml acetylated BSA and were stored at −20 °C. There was no measurable decrease in protein activity after 18 months storage at −20 °C. The concentrations of active EcoRV protein were determined by titration with the specific site 310 bp DNA fragment under conditions of virtually stoichiometric binding as described in (24, 27).

Single-turnover kinetics experiments measured by the self-cleavage assay

Specific EcoRV-DNA complexes were prepared by the incubation of EcoRV (~1.5 nM) with the 310 bp specific site DNA fragment (~3.3 nM) in the absence of divalent ions for a minimum of 3 hours at 20 °C to ensure that all protein present in the mixture is bound to its specific sequence in the enzymatically competent form (15). Solution conditions for the DNA-protein binding reaction were 20 mM imidazole buffer (pH 6.35, 6.88, or 7.4), 100 mM NaCl, 2 mM DTT, 50 μg/ml BSA, and 1.7 or 2.5 osmolal glycine betaine. We have demonstrated previously that under these conditions greater than 95% of all EcoRV added binds to its specific site on the 310-bp fragment at the pH range between 6.35 and 7.6 (15) or $K_a > 10^{10}\ M^{-1}$.

The basics of the self-cleavage technique is that the complex between restriction enzyme and specific DNA fragment pre-formed in the absence of divalent ions can be converted to the enzymatically active form by adding Mg^{2+} with only negligible protein dissociation before cleavage occurs. The cleavage mixture solution was adjusted to ensure final concentrations of ~4.0 μM specific site 30 bp oligonucleotide (1200 fold excess over specific fragment in molar concentration), 20 mM imidazole buffer (with same pH as in the corresponding DNA-protein pool), 100 mM NaCl, 2 mM DTT, 0.25–4 mM Mg^{2+}, and 2.5–5 osmolal glycine betaine in the final samples. The specific site oligonucleotide is added to bind any enzyme that dissociates from the DNA fragment both before and after cleavage. To start the cleavage reaction, 30 μl of the cleavage mix was added to 30 μl aliquots from the DNA-protein pool. Cleavage was allowed to proceed for different periods of time before the reaction was stopped by adding EDTA to a final concentration of 20 mM.

Control experiments were performed to measure the initial fraction of DNA with specifically bound EcoRV. For these experiments the composition of the cleavage mix was adjusted to ensure a final pH of ~7.0, 10 mM $MgCl_2$, 100 mM NaCl, and 1200-fold molar excess of specific site oligonucleotide competitor over the specific site fragment. Enough glycine betaine was added to the cleavage mixture to ensure a final total osmotic pressure of at least 2.5 osmolal. Samples were incubated for 10 minutes at 20 °C; the cleavage reaction was then stopped by adding EDTA to a final concentration of 20 mM. As was demonstrated previously (15) at these experimental conditions the fraction of DNA cleaved reflects the fraction of DNA initially bound to EcoRV.

DNA digestion products were purified using GenElute PCR Clean-up kit (Sigma Chemical Co., Inc.).

Gel Electrophoresis

Loading dye was added to the purified DNA digestion products to a final concentration of 5% glycerol and 0.05% bromphenol blue) 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 allowed to run at 350 V for 2.5–3 hours. The reaction products of supercoiled plasmid cleavage were loaded directly onto 1.3% agarose gels in TAE and run at 50 V for 18 hours.

Electrophoretic bands containing uncleaved and cleaved DNA fragments were stained with the fluorescent dye SYBR Green I (Molecular Probes). The gels were imaged with a Typhoon FLA-7000 Fluorescent Image Analyzer from GE. The FLA-7000 was interfaced to a Pentium PC. Band intensities were quantified using the Fuji Film software MultiGauge for Windows. Intensity data generated in MultiGauge was further analyzed using SigmaPlot 10.0 software (Systat Software Inc.); fractions of DNA cleaved were calculated for each time point. These fractions cleaved were normalized to the control for initially bound protein.

Kinetic and linkage analyses

The observed kinetics indicate at least two kinetic steps, with double stranded cleavage occurring only after the second step. A minimal model that satisfies the observed kinetics is depicted in Figure 1. After initiating cleavage with $MgCl_2$, the preformed DNA-protein complex (ES) can either proceed to an intermediate ES^* with rate constant k_1 or dissociate to E + S with rate constant k_d. The intermediate can then proceed to the double stranded cleavage product, P, with rate constant k_2. The kinetics equations associated with this scheme can be straightforwardly solved for the fraction cleaved to give:

$$\frac{[P]}{[ES]_0}=\frac{k_1(1-e^{-k_2 t})}{(k_1+k_d-k_2)}-\frac{k_1 k_2\left(1-e^{-(k_1+k_d)t}\right)}{(k_1+k_d)(k_1+k_d-k_2)}, \quad (1)$$

where $[ES]_0$ is the initial concentration of complex. There are two exponential relaxation rates: (k_1+k_d) for the loss of the initial substrate due to either reaction or dissociation and k_2 for the formation of product from the intermediate. The pre-exponential amplitudes are fixed by the relaxation rate constants. At long times, the fraction cleaved approaches a limiting value of $k_1/(k_1+k_d)$. The rate constants, k_1, k_2, and k_d were fit to the experimental data using Mathcad 14 software from Parametric Technology Corporation.

The same linkage reactions based on the Gibbs-Duhem equation that are used to relate changes in equilibrium binding constants to changes in pH, salt, and water activity can be applied to rate constants. Instead of the free energy differences of equilibrium constants, rate constants depend on activation free energies, $\Delta G_a^{\dagger}$. The difference in the number of H^+ ions, salt ions or water molecules, $\Delta N^{\dagger}$, associated with the initial state of a reaction step and its transition state can be determined from the dependence of the rate constant, k, on the activity, *a*, of each component in solution by,

$$\frac{d\log(k)}{d\log(a)}=\frac{d\ln(k)}{d\ln(a)}=-\frac{d\Delta G_a^{\dagger}}{kt\, d\ln(a)}=\Delta N_a^{\dagger} \quad (2)$$

This equation is specifically for the rate limiting step. Very fast binding reactions (compared to k_1 and k_2) of solution components that precede the slow step would not contribute to $\Delta N^{\dagger}$ values. The contribution from steady state equilibrium binding of solution components before the slow step would depend on the relative saturation of binding sites. If $\Delta N^{\dagger}$ is a constant over the concentration range examined, then Hill plots will give coefficients $n_H = \Delta N^{\dagger}$. For Mg^{2+}, $\log(a) = \log([Mg^{2+}])$; for pH, $\log(a) = -\text{pH}$; and for water, $\ln(a) = -\text{osmolal}/55.6$.

Results

Single-turnover EcoRV cleavage kinetic curves show a distinct lag-phase

Our single-turnover kinetic assay consists of three steps: the binding reaction, the cleavage reaction, and a stop reaction. In the binding reaction, EcoRV is incubated in the absence of divalent cofactor with a 310 bp DNA fragment containing a single EcoRV specific site for a long enough times to ensure that all protein is specifically bound to the DNA fragment (15). Typically the enzyme to recognition site ratio is 0.5–0.6. The cleavage reaction is initiated by adding Mg^{2+} and a 1200-fold molar excess of specific site 30 bp oligonucleotide to trap any free enzyme. The reaction is stopped at various times by adding EDTA. The fraction of fragment cleaved is determined by gel electrophoresis.

A typical kinetic experiment is illustrated in Figure 2. The solution conditions for the binding reaction were 20 mM imidazole pH 6.35, 100 mM NaCl, 1.7 osmolal glycine betaine, and 20° C. Under these experimental conditions, virtually all EcoRV (> 95%) is bound to its specific sequence at equilibrium (15). For the cleavage reaction, solution conditions were then adjusted to 1 mM Mg^{2+}, pH 6.35, 100 mM NaCl, ~4 μM specific site 30 bp oligonucleotide, and 3.0 osmolal glycine betaine. The cleavage reaction was stopped at various times by adding 20 mM EDTA and the DNA fragments were purified. Figure 2A shows a polyacrylamide gel of the purified DNA fragments. Increased cleavage is apparent with increased incubation times with Mg^{2+}. Figure 2B shows the time dependence of

cleavage. The ✰ symbol shows the fraction of initially bound protein. The initial fraction of specific complex is determined by a standard self-cleavage assay: the final reaction mix contains 10 mM $MgCl_2$, the pH is adjusted to ~ 7 with imidazole, and 3 osmolal glycine betaine. Under these conditions, virtually all initially protein-bound DNA fragments are cleaved after 10 minutes incubation; complex dissociation during this time is negligible (15, 28).

The fraction of DNA cleaved (Figure 2B) reaches a plateau only after about 180 min incubation of DNA-protein complex in the cleavage reaction mixture; the presence of a plateau is consistent with the expected behavior for a single-turnover reaction. Two prominent features are apparent in the kinetics curve. First, a distinct lag phase is observed at early times, indicating that the reaction involves two kinetic steps at minimum and that double-stranded cleavage happens only after the second step. Second, the plateau value for fraction DNA fragment cleaved is about 14% lower than the fraction that corresponds to the initial fraction of pre-bound fragment (star symbol on Fig. 2A and 2B) indicating that there is a weak but measurable dissociation of the pre-bound protein from the fragment without double-stranded cleavage during the time course of the experiment.

The formation of product, i.e. the fraction of DNA cleaved, can be straightforwardly calculated using equation 1, derived for the minimal reaction scheme shown in Figure 1. The solid line in Figure 2B shows the best fit of equation 1 to the data. Figure 2C shows the fit residuals. The average difference in fraction cleaved between the experimental points and the theoretical curve is < 0.004. The minimal model fits the data quite well. All experiments reported here were done at least in duplicate. The error in determining the rate constants from fitting the data for the three sets of experiments (varied Mg^{2+}, pH and water activity) ranged from 5 to 10% for k_2 and from 10 to 20% for k_1. Since dissociation was minimized, the error in k_d was significantly greater, but did not significantly affect k_1 or k_2.

In order to characterize the physical processes underlying k_1 and k_2, we now measure the dependence of these rate constants on $MgCl_2$ concentration, pH, and osmotic pressure.

Cleavage kinetics and Mg^{2+} concentration

The dependence of the rate constants k_1 and k_2 on Mg^{2+} concentration will give the number of Mg^{2+} ions that are coupled to each rate-limiting kinetic step. Figure 3A shows cleavage kinetics at 0.5, 1, and 2 mM $MgCl_2$, for pH 6.35 and 3.0 osmolal glycine betaine. The lines are fits to the data using equation 1. The theoretical fits describe the experimental data quite well for all $MgCl_2$ concentrations used. This dependence of the rate on Mg^{2+} concentration can be translated into a number of Mg^{2+} ions bound between the initial to transition states of the each reaction step, $\Delta N^{\dagger}_{Mg^{2+}}$,

$$\frac{d\log(k)}{d\log([Mg^{2+}])} = \Delta N^{\dagger}_{Mg^{2+}} \quad (3)$$

Figure 3B shows dependence of $\log(k_1)$ and $\log(k_2)$ on $\log[Mg^{2+}]$both at pH 6.35 with 3 osm glycine betaine and at pH 6.88 with 4 osm glycine betaine. The plots are reasonably linear over the entire range of $MgCl_2$ concentrations examined, 0.25 – 4 mM. Very similar numbers of metal ions are taken up at pH 6.35 and pH 6.88. At pH 6.35, both the k_1 and k_2 kinetic steps are coupled to the binding of 1.6 ± 0.1 Mg^{2+} ions. At pH 6.88, 2.0 ± 0.05 and 1.4 ± 0.1 Mg^{2+} ions are bound by the complex during first and second steps, respectively.

As given in Table 1, at pH 7.4 $\Delta N^{\dagger}_{Mg^{2+}}$ is 2.4 ± 0.2 and 1.3 ± 0.3 for the k_1 and k_2 kinetic

steps, respectively. Figure S1 of the Supplementary Materials shows that the data for k_1 at pH 6.88, for example, can clearly distinguish among $\Delta N^{\dagger}_{Mg^{2+}} = 1, 2, \text{ and } 3$.

Cleavage kinetics and pH

EcoRV cleavage kinetic curves were measured at different pH values ranging from 6.35 to 7.4 while keeping Mg^{2+} and glycine betaine concentrations constant. Figure 4A shows two sets of experimental data and corresponding fits for pH 6.35 (black circles) and pH 6.88 (open circles). Both curves were measured at 2 mM $MgCl_2$ and 4 osmolal betaine glycine. Under these conditions enzyme dissociation is less than 2% before cleavage occurs. Both k_1 and k_2 rates are significantly slower at pH 6.35 than at pH 6.88. Figure 4B shows the pH dependence of $\log(k_1)$ and $\log(k_2)$ at constant 0.5 mM Mg^{2+} and 3 osm glycine betaine. The pH dependence of the ratio of nonspecific and specific equilibrium constants, $K_{nsp\text{-}sp}$, determined previously (15) is also shown for comparison. The curves are reasonably linear over the pH range ~6.3–6.9 for $\log(k_1)$ and $\log(k_2)$ and from ~5.5–6.5 for $\log(K_{nsp\text{-}sp})$. The slope can be related to a difference in number of bound protons between the initial and transition states for each kinetic step by a standard linkage relation,

$$\frac{d\log(k)}{d\,pH} = -\Delta N^{\dagger}_{H^+} \quad (4)$$

The slope of the linear part of the $\log(K_{nsp\text{-}sp})$ dependence on pH is 2.0 ± 0.3 indicating that about two fewer H^+ ions are bound to the specific complex relative to the nonspecific complex. The k_1 and k_2 kinetic steps are coupled to the release of 2.3 ± 0.3 and 2.2 ± 0.4 H^+ ions, respectively. A total of ~ 4 H^+ ions are released by the protein as the complement of 3–4 Mg^{2+} ions for bound. Table 2 indicates that $\Delta N^{\dagger}_{H^+}$ is weakly dependent on Mg^{2+} concentration and osmotic pressure.

Cleavage kinetics and osmotic pressure

To characterize the conformational changes associated with the two kinetic steps, we measured the cleavage kinetics at different osmotic pressures or water activities set by the concentration of the neutral solute glycine betaine. A standard linkage relation can be applied to determine changes in hydration coupled to the conformational changes accompanying the kinetic steps.

$$\frac{d\ln(k)}{d\,[\,Osmolal]} = -\frac{\Delta N^{\dagger}_{w}}{55.5} \quad (5)$$

In this case, $\Delta N^{\dagger}_{w}$ represents changes both in sterically sequestered water and in the preferential hydration of solvent exposed surface area between the initial and transition states of each kinetic step (19, 25, 26).

Experimental kinetic curves measured at 3, 3.5, and 4 osmolal glycine betaine at constant 0.5 mM $MgCl_2$, pH 6.88 and their corresponding fits are shown in Figure 5A. The kinetic rate constants k_1 and k_2 decrease with increasing osmotic pressure (osmolal concentration). This dependence of reaction rates on osmotic pressure indicates that the conformational changes accompanying Mg^{2+} binding and H^+ release also take up water.

Figure 5B shows the dependence of the rate constants on glycine betaine osmolal concentration as prescribed by equation 5 both for pH 6.35 with 2 mM Mg^{2+} and for pH

6.88 with 0.5 mM Mg^{2+}. The four curves are all reasonably linear and parallel. The slopes range from 0.48 to 0.58, corresponding to the thermodynamic binding of some 30 ± 7 extra water molecules associated with the conformational changes leading to the transition state of each kinetic step. As seen in Table 3, this number of water molecules does not significantly depend on pH or Mg^{2+} concentration over the range examined.

The k_2 rate constant measured at pH 7.4, 1 mM Mg^{2+}, 5 osmolal betaine, and 20 °C is 0.32 min^{-1}. At pH 7.4 ~ 35 water molecules are coupled to each kinetic step. If this rate is extrapolated to no added osmolytes, the expected rate constant is ~ 0.15 sec^{-1}. This is less than two-fold slower than the rate reported by Baldwin et al (14) for 1 mM Mg^{2+}, pH 7.5, and a slightly higher temperature, 25 °C (their figure 5B).

Mg^{2+} binding is coupled to single strand cleavage

Halford and coworkers (30, 31) showed that supercoiled plasmid DNA is cleaved by EcoRV by successive single strand cuts. The first single strand cut relaxes the supercoil and second linearizes it. The three species can be separated on agarose gels. We have repeated these experiments using osmotic stress in order to show that Mg^{2+} binding is coupled to single strand cleavage. Figure 6A shows an agarose gel illustrating the time course of supercoiled plasmid cleavage by EcoRV at pH 6.35, 4 mM Mg^{2+}, 3 osmolal betaine, and 20 °C. Figure 6B shows the fractions of supercoiled, nicked, and linear plasmid as a function of time. The fraction linear plasmid shows an initial lag and the fraction nicked, relaxed plasmid goes through a maximum before decreasing. The fraction linear plasmid was fit to equation (1) that was used to fit double stranded cleavage of the linear DNA fragment. The fit is shown as the solid line through the data. Using the fit parameters from the linear plasmid, expected curves for the fractions of supercoiled and nicked plasmid can be calculated and are shown as the solid lines in the figure. The kinetic scheme used fits all the data quite well. For both pH 6.35 and 7.4 (data not shown), the k_1 rate constants are about 2–3 fold slower for plasmids than for the 350 bp linear fragments under the same experimental conditions. This might be due to supercoiling energetics slowing the reaction. The k_2 rate constants, however, are almost identical for plasmid and DNA fragment under the same experimental conditions suggesting that supercoiling is relaxed after the first nick even though the enzyme is still bound.

The Mg^{2+} and osmotic pressure dependences of the k_1 and k_2 rates were determined for pH 6.35. The Mg^{2+} dependence shown in figure 7A indicates that each step is coupled to the binding of 1.6 Mg^{2+} ions. This is almost identical to the DNA fragment results shown in Figure 3B and Table 1. The rate constant dependences on osmotic pressure are shown in figure 7B. About 40 water molecules are seen linked to each kinetic step, ~ 10 water molecules more than seen for the DNA fragment at pH 6.35. Mg^{2+} binding and single strand cleavage are tightly coupled.

Discussion

Since previous cleavage kinetic experiments (12, 14, 32) have given ambiguous results for the number of Mg^{2+} ions needed for cleavage by EcoRV, we have devised our own single-turnover protocol that incorporates osmotic stress to minimize complex dissociation before cleavage and to slow the overall rate of cleavage. We have used osmotic stress previously to stabilize protein-DNA complexes for biophysical and biochemical analysis. Dissociation reactions typically require binding extra water molecules. This can be made energetically even less favorable by applying osmotic pressure, thus slowing dissociation considerably (15, 19, 25, 28). In our view, ideal single-turnover experiments should bracket as few kinetic steps as possible. In our scheme, the reaction is initiated with virtually all enzyme bound to its recognition DNA sequence at the osmotic pressures used. Neither the DNA binding nor

the product dissociation steps are part of the probed reaction. The initial complex has no bound Mg^{2+}. The cleavage reaction is then started by adding Mg^{2+} ions. The small fraction of enzyme that does dissociate before cleavage (always < 20%) and any enzyme that is released after DNA fragment cleavage is trapped by the large molar excess of specific site oligonucleotide that is added along with the Mg^{2+}, preventing rebinding to the DNA fragment that is monitored for cleavage. We can assess the amount of initial complex that does dissociate before cleavage with a control reaction under solution conditions that effectively prevent dissociation. Single-turnover rates are typically measured on a very fast time-scale of milliseconds-seconds (14, 30, 33). By using osmotic pressure however, the cleavage kinetics of EcoRV are slowed enough to allow for measurements on a time scale of minutes to hours.

We use glycine betaine as the osmolyte in the kinetic experiments presented here. Glycine betaine is one of the most abundant natural non-perturbing solutes used *in vivo* to protect cells from high osmotic stress. This compatible osmolyte is found in a wide variety of bacteria, plants, marine animals, and the mammalian renal medulla (34, 35). DNA-protein complexes can withstand extremely high concentrations of glycine betaine without damage. Using kinetic studies, we have shown earlier that the difference between water retained by the specific and nonspecific DNA-EcoRI restriction enzyme complexes is constant up to 6 osmolal glycine betaine (26). This result indicates that both complexes maintain their conformations up to very high osmotic pressures. The available evidence indicates that glycine betaine simply acts osmotically on the EcoRV-DNA complexes as well, at least up to 5 osmolal glycine betaine (highest solute concentration used in this work). Using competition equilibrium studies we confirmed that the difference in water sequestered by the specific and nonspecific DNA-EcoRV complexes remains unchanged up to the highest concentration examined, 3 osmolal glycine betaine (data not shown). Additionally, the linearity of plots shown in Figure 5B indicates that glycine betaine acts osmotically on the cleavage rates, k_1 and k_2, measured over the range of concentrations 2.5 – 5 osmolal. Extrapolation of the k_2 rate constant measured at pH 7.4, 1 mM Mg^{2+}, and 5 osmolal betaine to no betaine is within a factor of two of the equivalent rate constant measured by Baldwin et al (14) under similar experimental conditions. This is further strong indication that glycine betaine, even at quite high osmolality, is only acting osmotically. An additional utility of using glycine betaine as an osmolyte is that it does not significantly change activity of either Na^+ or Mg^{2+} ions up to 5 osmolal glycine betaine (confirmed using ion selective electrode, data not shown). The pK of the imidazole buffer used is additionally insensitive to glycine betaine also up to high solute concentrations.

As illustrated in Figure 2B, the fraction DNA cleaved by EcoRV reaches a plateau after about 180 min of DNA-protein complex incubation with the cleavage mix. The presence of the plateau indicates that only the DNA fragments that were pre-bound by protein were cleaved throughout the duration of the experiment. The plateau value of the cleaved fraction is somewhat smaller than the fraction of initially bound fragment, indicating that a small fraction of the initially bound enzyme dissociates before cleavage even with osmotic pressure. The kinetic curves of DNA cleavage clearly have a lag phase indicating at least two consecutive steps are required for the reaction and that cleavage only occurs after the second step. The minimal model depicted in Figure 1 with three rate constant, k_1, k_2, and k_d, fits the data quite well as is confirmed by the fit residuals shown in Figure 2C and the agreement between experiment and equation 1 seen in figures 2B, 3A, 4A, 5A, and 6A. A similar lag phase for cleavage kinetics has been seen by others (14, 30, 31). A dissociation reaction from the intermediate, ES^* in Figure 1, was not included since it is generally thought that divalent ion binding strengthens specific sequence binding (11, 36–38).

Each of the kinetic steps characterized by k_1 and k_2 could potentially represent a set of smaller steps, but the observation that the kinetics can be well fit with the two exponentials suggests that there is only one rate-limiting step that dominates each rate constant. In order to characterize the physical processes underlying rates k_1 and k_2, we measured dependence of these individual rate constants on $MgCl_2$ concentration, pH, and osmotic pressure.

The Mg^{2+} concentration dependence of k_1 and k_2 is summarized in Table 1 for three pH values. Both k_1 and k_2 are coupled to the binding of Mg^{2+} ions. This is in contrast to the results of Baldwin et al (14) who observed either a net release of Mg^{2+} or no Mg^{2+} dependence of the lag phase kinetics through ~ 4 mM Mg^{2+}. We have examined the conditions used by Baldwin et al. (14) and found that enzyme dissociation is faster than cleavage without osmotic stress (Supplementary material). The lag phase observed by Baldwin et al. incorporates the kinetics of dissociation, binding of Mg^{2+} to free enzyme, the subsequent rebinding to the DNA, and only then will cleavage occur. The lag we observe is due to a kinetic step between the initially bound enzyme and final cleavage step without dissociation. The plots shown in Figure 3B are all quite linear indicating that the number of Mg^{2+} bound per enzyme coupled to k_1 and k_2 is constant over the Mg^{2+} concentration range measured, 0.25 to 2 mM for pH 6.88 and from 0.5 to 4 mM for pH 6.35. The average number bound per enzyme linked to k_1 increases from 1.6 at pH 6.35 to 2.4 at pH 7.4. The increase in $\Delta N^{\dagger}_{Mg^{2+}}$ per enzyme with increasing pH for k_1 is balanced by a decrease in $\Delta N^{\dagger}_{Mg^{2+}}$ for k_2, from 1.6 at pH 6.35 to 1.3 at pH 7.4. The total $\Delta N^{\dagger}_{Mg^{2+}}$ per enzyme increases from 3.2 at pH 6.35 to 3.7 at pH 7.4. We conclude that at pH 7.4 EcoRV requires close to 4 Mg^{2+} ions for double stranded cleavage of DNA even at Mg^{2+} concentrations as low as 0.25 mM. The decrease in $\Delta N^{\dagger}_{Mg^{2+}}$ coupled to k_2 toward 1 with increasing pH is consistent with the observations of Jeltsch et al (32) and Baldwin et al (14) that k_{cat} is linked to ~ 1 Mg^{2+} per enzyme at pH 7.5. It is not clear if the smaller total $\Delta N^{\dagger}_{Mg^{2+}}$ values at the lower pH values are, for example, due to a mix of 1 and 2 Mg^{2+} per monomer in the cleavage reaction. Another possible complication is that the Mg^{2+} concentration in the vicinity of the enzyme-DNA complex may not be linearly proportional to the bulk concentration due to the electrostatic interaction of Mg^{2+} with the negatively charged DNA. It is also possible that a small fraction of the initially specifically bound enzyme does undergo a specific to nonspecific transition, binds Mg^{2+}, then rebinds specifically to the recognition sequence before dissociating from the DNA fragment. Depending on the relative rates of these steps, these Mg^{2+} ions may not contribute to the total number observed.

EcoRV enzymatic cleavage activity decreases dramatically as pH decreases from 7.5 to 6.0 while, paradoxically, the specific equilibrium binding and binding specificity in the absence of Mg^{2+}, K_{rel}, strongly increase (15, 36). It has been proposed (33, 37, 39–41) that both the decreased enzymatic activity and the increased specific binding at lower pH values are due to titration of acidic Glu and Asp residues in the catalytic site of the specific, but not the nonspecific, complex. This neutralization of the Glu and/or Asp residues would not only lower Mg^{2+} binding affinity, but also decrease the repulsive electrostatic energy from placing negative DNA –phosphate charges in the active site of the specific complex in the absence of divalent ions. The pKa values of these Glu and Asp groups seem to be considerably shifted due to the high density of negative charges in the active site with specifically bound DNA. The pH dependence of K_{rel} results from the difference in these pKa values between the specific and nonspecific complexes. The pH dependence of the single-turnover cleavage kinetics in the range 6.35 – 6.9 with constant 0.5 mM Mg^{2+} and 3 osmolal glycine betaine showed that both the k_1 and k_2 kinetic steps are coupled to the release of nearly the same number of hydrogen ions, $\Delta N^{\dagger}_{H^+}$ ~2.3 and ~2.2 H^+ ions,

respectively (Figure 4B). A total of ~ 4 H^+ ions are released in this pH range by the EcoRV-DNA complex in the process of binding the Mg^{2+} ions resulting in double-stranded cleavage. The binding of Mg2+ would be expected to shift the pKa of the Asp and Glu residues in the active site to more normal values resulting in the release of ~2 H^+ ions/dimer for pH values in the 6.5 range. Our previous equilibrium competitive binding experiments in the absence of Mg^{2+} indicated that about two more H^+ ions are bound by the specific complex relative to the nonspecific complex (15) for the pH range between 6.0 and 6.6 or one per monomer. The second H^+ ion released per monomer linked to k_1 and k_2 could be from the Mg^{2+} activated water that hydrolyzes the DNA phosphate bond. These results suggest that there is a binding competition between Mg^{2+} and H^+ ions in the active center of the specific EcoRV-DNA complex in the pH range 6.35 – 6.9. The Mg^{2+} binding constant of the specific complex decreases with decreasing pH. The pH dependences of k_1, k_2, and K_{rel} is much smaller for pH values greater than ~ 7, indicating that at higher pH the active site Glu and Asp residues are likely fully charged.

Conformational changes are often accompanied by changes in water accessible surface areas. We have previously employed the osmotic stress technique (15, 16, 19, 24–27, 42, 43) to measure changes in hydration coupled to conformational differences from the dependence of equilibrium constants or kinetic rates on osmotic pressure (water activity). As with $\Delta N^{\dagger}_{H^+}$ and $\Delta N^{\dagger}_{Mg^{2+}}$, values of $\Delta N^{\dagger}_{w}$ linked to k_1 and k_2 (Table 3) are quite similar, averaging ~28–35 extra water molecules for each kinetic step, and are fairly insensitive to pH and Mg^{2+} concentration. The dependence of solution viscosity on glycine betaine concentration (25) indicates that 5 of the waters may be due to an increase in viscosity rather than an increase in sequestered water. The increased viscosity could slow the conformational transition. The complex binds at least 23–30 extra water molecules that exclude osmolyte in going from the initial conformation to the transition state. As was pointed out by Baldwin et al (14), the active site in the crystal structure of the specific EcoRV-DNA complex in the absence of Mg^{2+} (44) is not readily accessible to Mg^{2+}. A conformational change is necessary for Mg^{2+} binding. We had initially anticipated that each step might be coupled to a rigid specific – nonspecific transition at the recognition site (15). This transition, however, is coupled to ~120 waters, about 4–5 fold greater than is observed. The conformational changes coupled to k_1 and k_2 are more subtle. This smaller conformational change would allow Mg^{2+} binding with less chance of complex dissociation than the specific-nonspecific transition. Since we have only used one osmolyte, glycine betaine, we cannot determine if the $\Delta N^{\dagger}_{w}$ observed linked to the conformational change is due to changes in sterically sequestered water or to changes in water exposed surface area.

The two kinetic steps have strikingly similar values of $\Delta N^{\dagger}_{H^+}$, $\Delta N^{\dagger}_{Mg^{2+}}$, and $\Delta N^{\dagger}_{w}$. We propose that each step involves the conformational change of a single enzyme monomer that allows Mg^{2+} binding and H^+ release. Using a supercoiled plasmid, we showed that single stranded cleavage observed by Halford and coworkers (30, 31) results from the binding of the first set of Mg^{2+} ions. In contrast, however, the binding of a complement of 2 Mg^{2+} ions to one enzyme monomer of the specific complex in the crystal (8) did not result in cleavage. Given the binding of extra ~30 water molecules coupled to Mg^{2+} binding to each monomer, it is remarkable that a first set of Mg^{2+} ions could bind in the constrained crystalline environment and could explain why the second subunit remained Mg^{2+}-free in the crystal (8). At pH 6.35, the dependences of k_1 and k_2 on Mg^{2+} concentration, pH, and osmolyte concentration are almost exactly identical. For this condition, the ratio of k_1/k_2 is ~ 2.8 ± 0.5; for independent and identical kinetic steps this ratio would have a statistical value of 2. This result suggests an interaction between the two subunits that causes the Mg^{2+} binding by the second subunit to be slightly more difficult. The increase in Mg^{2+} ions coupled to the k_1

step with increasing pH (from 1.6 at pH 6.35 to 2.4 at pH 7.4) suggests that one monomer may be able to share divalent ions with the other.

Cellular concentration of free Mg^{2+} has been estimated to be in the 0.5 – 4 mM range (45, 46), significantly lower than 10 – 30 mM $MgCl_2$ routinely used for *in vitro* assays. The kinetic results of Jeltsch et al (32), Baldwin et al (14), Groll et al (12), Pingoud et al (5), and Xie et al (11) all indicate that most free restriction nucleases will not have a full complement of 4 Mg^{2+} ions generally considered necessary to cleave DNA efficiently even at fairly high Mg^{2+} ion concentrations. It is possible that only that fraction of free enzyme with a full complement of 4 Mg^{2+} ions can bind and cleave DNA efficiently, i.e., that enzyme without a full Mg^{2+} complement can only bind nonproductively or bind and cleave DNA at a much lower rate. Alternatively, specifically bound enzyme with less than a full complement of Mg^{2+} ions can bind the extra Mg^{2+} needed while bound and then cleave DNA efficiently. We see here that specifically bound EcoRV is quite capable of binding Mg^{2+} and cleaving DNA without first dissociating. Indeed the binding constant of Mg^{2+} to the complex is much larger than to free enzyme. The association binding constant of EcoRV with bound divalent ions to its DNA recognition site is estimated as at least ~100-fold larger than for the metal-free enzyme (37). This also means that divalent ion binding to the preformed DNA-enzyme specific complex is ~ 100-fold stronger than to the free enzyme.

Conclusions

Our single-turnover assay includes only the kinetic steps between a specifically bound complex without any Mg^{2+} and the double stranded cleavage product. In particular, neither the binding of free enzyme nor the dissociation of the cleaved product contributes anything to the monitored reaction. The kinetic curves show two steps in the cleavage reaction. The dependence of the rate constants for the two steps on solution conditions indicates that ~ 4 Mg^{2+} ions per dimer are bound by the enzyme at pH 7.4 even at 0.25 mM Mg^{2+}. The 2 Mg^{2+} per monomer requirement for cleavage has been experimentally verified. In the pH range 6.35 to 6.9, ~ 4 H^+ protons per dimer are released in total, likely due to titrations of aspartate or glutamate residues in the catalytic sites and the formation of Mg^{2+} activated hydroxides that cleave that hydrolyze the DNA phosphate backbone. The conformation change underlying each kinetic step entails the binding of ~30 water molecules. The symmetry of the dependence of the two kinetic steps on solution conditions suggests that each monomer acts independently to bind the Mg^{2+} needed for the cleavage reaction and cleave a single strand. The use of osmotic stress in these experiments was critical for these measurements. The approach we developed and described here might be useful for exploring the cleavage mechanisms of other metal ion dependent nucleases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EDTA	ethylenediaminetetraacetic acid
BSA	bovine serine albumin
DTT	dithiothreitol
TMAO	trimethylamine oxide

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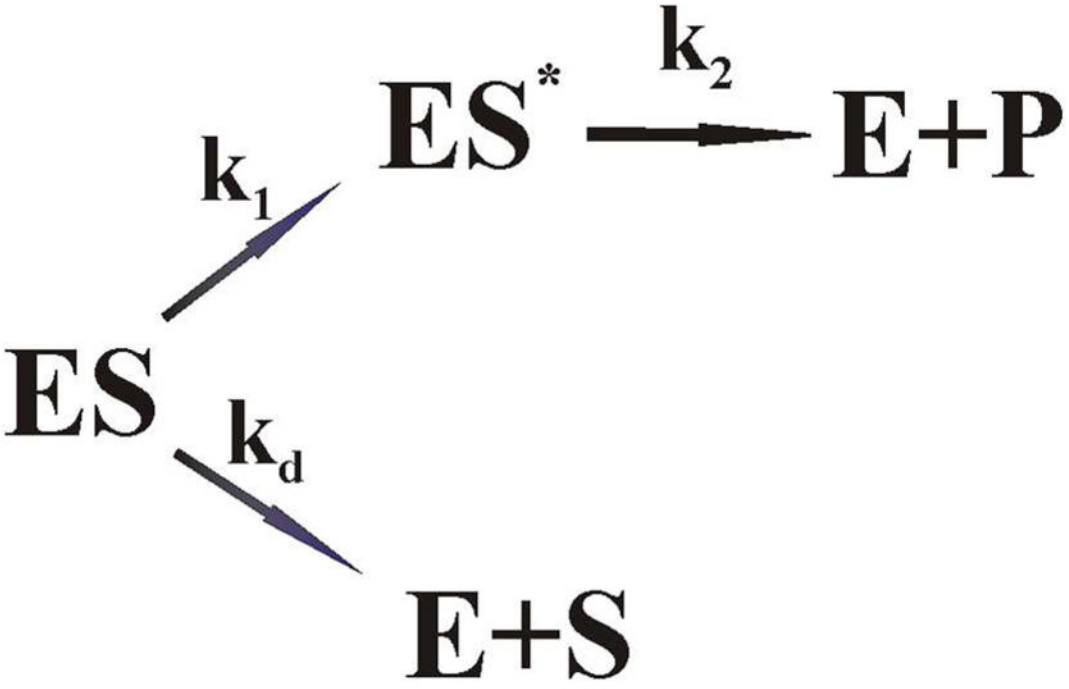

Figure 1. A minimal model for Mg^{2+} binding in the EcoRV cleavage reaction that satisfies the observed kinetics

Starting from the specifically bound conformation (ES), EcoRV can proceed to an intermediate form (ES^*) with rate constant k_1 or the complex can dissociate from the DNA (E + S) with rate constant k_d. From the intermediate, EcoRV can produce a cleaved product (P) with rate constant k_2. Once EcoRV has reached the intermediate form, ES^*, dissociation of the complex is unlikely.

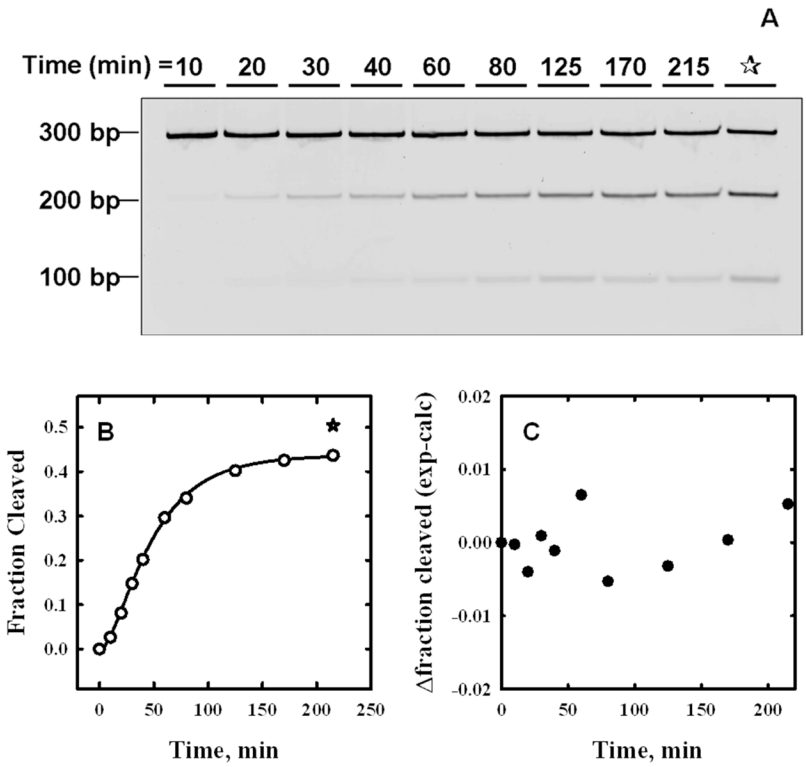

Figure 2. Single turn-over experiments were performed to measure EcoRV cleavage kinetics

(A) A gel image illustrating the results of the cleavage kinetics experiment is shown. Cleavage of the 310 bp fragment gives the two fragments that are about 100 and 200 bp in length. Fractions of cleaved DNA fragment were measured at the indicated time points under reaction conditions of 1 mM $MgCl_2$, ~4 μM specific site 30 bp oligonucleotide, 100 mM NaCl, 3.0 osmolal glycine betaine, and 20 mM imidazole buffer pH 6.35. Reactions were incubated at 20 °C. The oligonucleotide is added to trap any free enzyme. The star symbol designates an experimental control for the fraction of DNA bound by EcoRV in the initial DNA-protein pool which was pre-incubated in the absence of Mg^{2+}. The fraction of DNA cleaved for the control point was obtained under the same conditions as described above except that 10 mM $MgCl_2$ and imidazole buffer of pH 7.0 were used in the final reaction mixture. The control sample was incubated with the cleavage mix for 10 min. At these conditions, the fraction of DNA cleaved reflects the fraction of DNA initially bound. (B) The time-dependence of the fraction of DNA cleaved by EcoRV calculated from the gel displayed in (A) is shown. The curve demonstrates a distinct lag phase suggesting the reaction occurs in at least two steps. The solid line is the best fit obtained using the minimal model (Figure 1 and Equation 1) with parameters: k_1=0.064 min^{-1}, k_2=0.025 min^{-1}, k_d=9.9×10^{-3} min^{-1}. (C) The differences in the fraction cleaved for the experimental points and the theoretical curve at each time point reveals less than 2% differences between the values confirming that the minimal model provides an adequate fit to the experimental data.

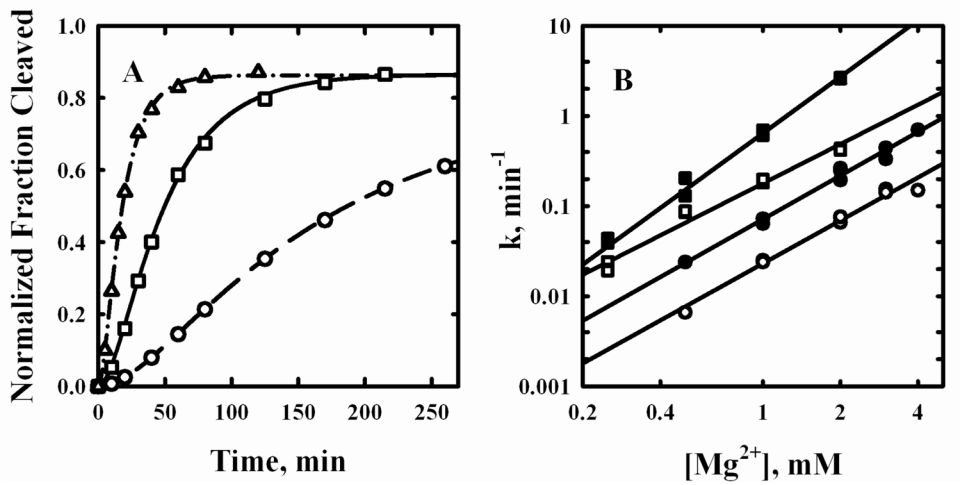

Figure 3. Cleavage kinetics and Mg^{2+} concentration

(A) The rates of single turnover DNA cleavage were measured at 0.5 (○), 1.0 (□), and 2.0 (△) mM $MgCl_2$, 3.0 osmolal glycine betaine, 100 mM NaCl, and 20 mM imidazole buffer pH 6.35. Lines correspond to the best fits obtained using Equation 1. (B) Parameters k_1 and k_2, derived from the set of experiments analogous to those shown in (A) at pH 6.35 (●– k_1, ○ – k_2) and pH 6.88 (■ – k_1, □ – k_2) exhibit a linear dependence on Mg^{2+} concentration on a log-log scale. The slopes calculated from the linear regressions of these plots can be translated into a number of Mg^{2+} ions associated with the particular kinetic step using Equation 3. For pH 6.35, slopes for k_1 and k_2 correspond to 1.6±0.1 and 1.6±0.1 Mg^{2+} ions, respectively, whereas at pH 6.88, slopes for k_1 and k_2 correspond to 2.0±0.06 and 1.4±0.09 Mg^{2+} ions, respectively. Experiments were performed at pH 6.35, 0.5–4 mM $MgCl_2$ and 3 osmolal glycine betaine, or at pH 6.88, 0.25–2 mM $MgCl_2$ and 4 osmolal glycine betaine. All other solution conditions were as outlined in Figure 2 caption.

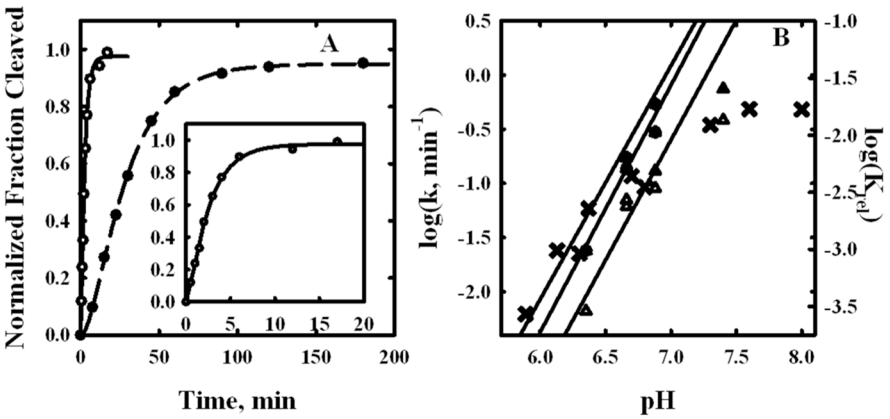

Figure 4. Cleavage kinetics and pH

(A) Single turn-over cleavage experiments were performed at pH 6.35 (●) and pH 6.88 (○), 2 mM $MgCl_2$, and 4 osmolal glycine betaine. The lines correspond to the best theoretical fits obtained using Equation 1. The figure inset shows an expanded view of the kinetics at pH 6.88. (B) The pH dependence of the rate constants k_1 (▲) and k_2 (△) derived from the set of kinetic experiments analogous to those shown in (A), is shown here. Kinetic experiments were performed at 0.5 mM $MgCl_2$, 3 osmolal glycine betaine, and pH range 6.35–7.4. The pH dependence of the equilibrium competition nonspecific versus specific EcoRV binding constants, $K_{sp\text{-}nsp}$ (✖), was obtained previously (15) and is shown for the comparison. The slope of the linear portions of each curve on log-log scale can be translated to a number of protons associated with the corresponding kinetic step or with the difference in number of protons associated to the formation of the specific versus nonspecific complex. The linear regions of k_1, k_2, and $K_{nsp\text{-}sp}$ pH dependencies correspond to 2.3±0.3, 2.2±0.4, and 2.0±0.3 protons, respectively. Other solution conditions were as described in the caption to Figure 2.

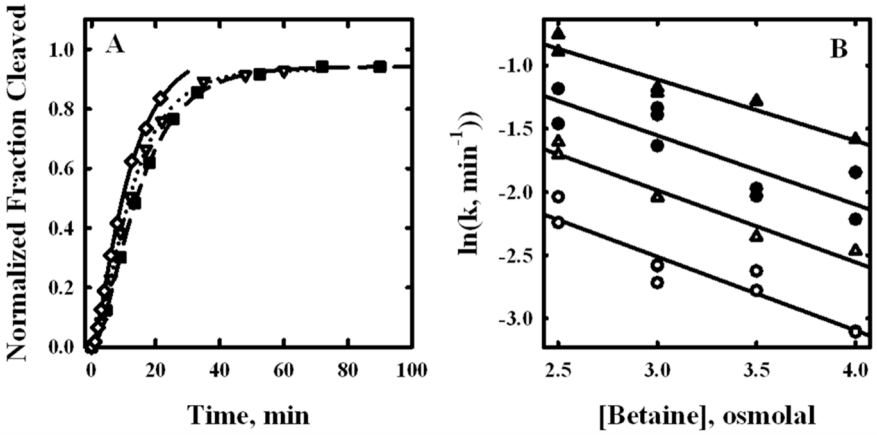

Figure 5. Cleavage kinetics and osmotic pressure

(A) Kinetic curves for three experiments performed at pH 6.88, 0.5 mM $MgCl_2$, and osmotic pressures of 3 (◇), 3.5 (▽), and 4 (■) osmolal glycine betaine are shown. At 3 osmolal betaine, k_1= 0.5382 min^{-1}; k_2= 0.0902 min^{-1}; whereas at 4 osmolal betaine, k_1=0.2046 min^{-1}, k_2=0.0851 min^{-1}. (B) The rates from the curves analogous to those shown in (A) measured at pH 6.35 (● - k_1, ○ - k_2) and 6.88 (▲ - k_1, △ - k_2) vary linearly with osmotic pressure. At pH 6.35 the slopes for k_1 and k_2 are 0.54±0.1 (corresponding to an uptake of ~30 waters) and 0.58±0.08 (32.2 waters), respectively, while at pH 6.88, slopes for k_1 and k_2 are 0.48±0.07 (26.7 waters) and 0.57±0.07 (31.7 waters). Other conditions were as described in Figure 2 caption.

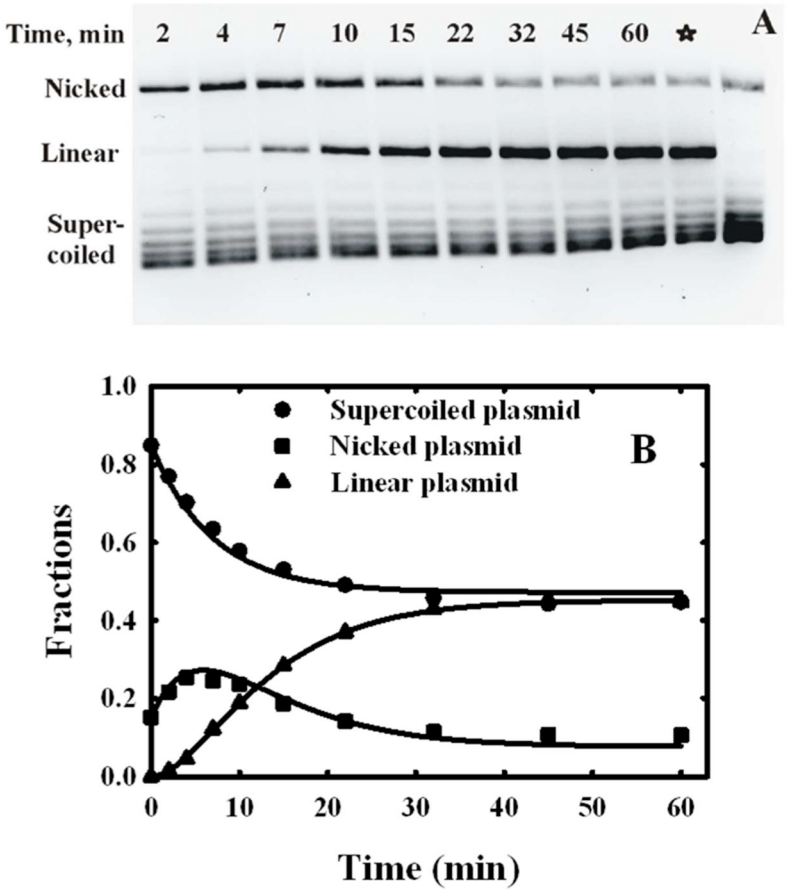

Figure 6. Cleavage kinetics of supercoiled plasmid

(A) A gel image illustrating the cleavage kinetics of supercoiled plasmid is shown. The plasmid Litmus28i was pre-incubated with EcoRV in 100 mM NaCl, pH 6.35, and 3 osmolal glycine betaine at 20°C in the absence of Mg^{2+}. The cleavage reaction was initiated by adding reaction mix to bring the final Mg^{2+} concentration to 1 mM with a 1600-fold molecular excess of specific site oligonucleotide while maintaining the 100 mM NaCl, pH 6.35, and 3 osmolal glycine betaine. At various times aliquots were taken and the reaction stopped by adding EDTA. The products were separated on a 1.3% agarose gel. Reaction times increase from left to right. The lane marked with the ☆ was the control experiment for total enzyme initially bound to plasmid. The last lane on the right is untreated plasmid. The bands for nicked, relaxed circle, linear plasmid, and supercoiled DNA are labeled. Note the initial increase then subsequent decrease in the intensity of the relaxed circle band. (B) The relative fractions of nicked (■), linear (▲), and supercoiled (●) DNA were determined from staining intensities and are shown as a function of time. The linear DNA data was fit with equation 1 and is shown as the line through the data. Using the parameters determined from the fit to the linear DNA (k_1 = 0.13 min^{-1}, k_2 = 0.14 min^{-1}, and k_d = 0.012 min^{-1}) curves for nicked and supercoiled DNA are shown. The fractions were calculated assuming that k_1 corresponds to the first single strand cleavage and k_2 to the second. The kinetic formalism describes the data quite well. Mg^{2+} binding is coupled to single strand cleavage.

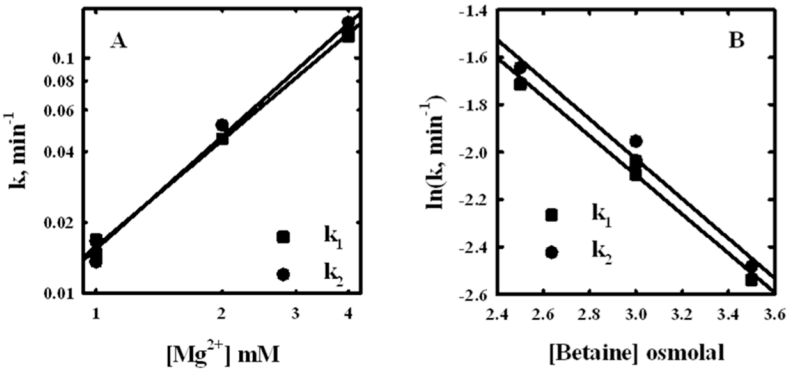

Figure 7. Dependence of supercoil cleavage kinetics on Mg^{2+} concentration and osmotic pressure

(A) The dependence of k_1 and k_2 on Mg^{2+} concentration for pH 6.35, and 3 osmolal betaine at 20 °C is shown as a log-log plot as in figure 3B. For both rate steps, $\Delta N^{\dagger}_{Mg^{2+}} = 1.6 \pm 0.4\,Mg^{2+}$ Mg^{2+} ions. This is virtually identical to the results for the DNA fragment under the same experimental conditions. Unlike the DNA fragment results, values of k_1 and k_2 are closely similar. (B) The dependence of ln(k) on glycine betaine osmolality is shown for k1 and k2 as in figure 5B. For both reaction steps, $\Delta N^{\dagger}_{w} = 42 \pm 6$ water molecules, about 10 more waters than observed for the DNA fragment.

Table 1

The number of Mg^{2+} ions ($\Delta N^{\dagger}_{Mg2+}$) associated with each kinetic step of the EcoRV cleavage reaction are shown for three different pH and osmotic pressure values.

		$\Delta N^{\dagger}_{Mg2+}$		
pH	**Betaine Osmotic Pressure**	**k_1**	**k_2**	**Total**
6.35	3 osmolal	1.6±0.1	1.6±0.1	3.2±0.2
6.88	4 osmolal	2.0±0.06	1.4±0.09	3.4±0.15
7.40*	5 osmolal	2.4±0.2	1.3±0.3	3.7±0.5

*The k_1 and k_2 at pH 7.4 were fit using data points combined from multiple experiments.

Table 2

The number of protons ($\Delta N^{\dagger}_{H+}$) associated with each kinetic step of the EcoRV cleavage reaction are shown for three different osmotic pressure and Mg^{2+} concentrations.

		$\Delta N^{\dagger}_{H+}$		
Betaine Osmotic Pressure	**[Mg^{2+}]**	**k_1**	**k_2**	**Total**
3 osmolal	0.5 mM	2.3±0.3	2.2±0.4	4.5±0.7
4 osmolal	2.0 mM	2.4±0.08	1.8±0.05	4.2±0.13
3 osmolal	4.0 mM**	2.6	1.9	4.5

** Only two points measured; MES buffer was used at pH 6.0 and imidazole was used at pH 6.35.

Table 3

The number of water molecules ($\Delta N^{\dagger}_{w}$) associated with each kinetic step of the EcoRV cleavage reaction are shown for three different Mg^{2+} concentrations and pH values.

		$\Delta N^{\dagger}_{w}$		
pH	**[Mg^{2+}]**	**k_1**	**k_2**	**Total**
6.35	2 mM	30±7	32±5	62±12
6.88	0.5 mM	27±8	32±6	59±14
7.40*	0.5 mM	35±9	36±3	72±12

*The values of k_1 and k_2 at pH 7.4 were fit using data points combined from multiple experiments.