BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

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

Rocco Ferrandino, Nina Sidorova, and Donald Rau*

The Program in Physical Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, United States

Supporting Information

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 has not been established. In this work, we employ a single-turnover technique that uses osmotic stress 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 time scale 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 cofactors 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 and thereby protecting bacteria from the invasion of foreign DNA. Despite the wealth of structural and biochemical information available about the role of metal ions in the cleavage reactions (reviewed in refs 1−4), the exact number of Mg^{2+} ions 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] Binding of divalent metal ion to three distinct sites per monomer has been seen in different crystals of EcoRV complexes,[6−9] but the three sites 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 by proposing that the two Mg^{2+} ions shift among the 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 by 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 rate of cleavage 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 to be 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 a fixed Mn^{2+} or Mg^{2+} concentration, Vipond et al.[10] found that the 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 auxiliary 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 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 that for the case in which two metal

Received: August 9, 2013
Revised: November 14, 2013
Published: December 16, 2013

dx.doi.org/10.1021/bi401089y | *Biochemistry* 2014, 53, 235−246

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 a 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 level reduced compared to that of 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 the 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 ensuring 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. The tryptophan fluorescence was used to monitor EcoRV conformational changes. Adding Mg^{2+} to EcoRV already bound to DNA produced 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 of 0.25−6 mM. The Mg^{2+} dependence of the first step associated with the lag phase was more complicated. For Mg^{2+} concentrations of 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 higher 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^+ atoms, and waters 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 cofactors. We also found that in addition to metal cofactors, 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 for the specific 310 bp long DNA fragment and the nonspecific 30 bp long oligonucleotide is only ∼56, but this ratio increases to $\sim 2.4 \times 10^3$ in the presence of 1.7 osmolal betaine glycine. The specific sequence binding constant, K_a, increases from $\sim 2 \times 10^8$ to $\sim 2 \times 10^{10}$ 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 $\sim 1.2 \times 10^3$ in the absence of solute; in the presence of 1.7 osmolal glycine betaine, the ratio increases to $\sim 4.5 \times 10^4$. This large increase occurs 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 the water activity. This sensitivity to osmotic pressure is a common feature of many protein−DNA complexes.[15−27] Because 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 the 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 a high degree of 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 because 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 described well 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 of 6.0−6.8, 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+} ions needed for cleavage by EcoRV and cleavage 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 ref 28. Briefly, the 533 bp DNA fragment containing a single EcoRV recognition sequence was isolated from the SphI and HindIII digestion of the pBR322 plasmid using standard techniques. The 310 bp fragment was then obtained from the 533 bp fragment using polymerase chain reaction (PCR) with internal primers. Cleavage of the 310 bp fragment at the EcoRV recognition site yields 107 and 203 bp DNA fragments. The pBR322 plasmid and restriction enzymes were ordered from New England Biolabs. The primer oligonucleotides were purchased from Invitrogen. Supercoiled plasmid Litmus28i was purchased from New England Biolabs and used without further purification.

The sequence of the double-stranded 30 bp 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-HCl (pH 7.5), and 1 mM EDTA]. Double-stranded 30 bp oligonucleotides containing the EcoRV-specific cleavage site were prepared as described in refs 15 and 27. After annealing and purification using P6 Bio-Spin columns, double-stranded oligonucleotides were precipitated with ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 7.5) and 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^{-1} mM^{-1} base $pairs^{-1}$ at 260 nm. Absorption spectra were recorded 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] previously described in ref 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, and 200 μg/mL acetylated BSA and were stored at −20 °C. There was no measurable decrease in activity after the protein had been stored for 18 months 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 refs 24 and 27.

Single-Turnover Kinetic 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 h at 20 °C to ensure that all protein present in the mixture was 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 more than 95% of all EcoRV added binds to its specific site on the 310 bp fragment in the pH range between 6.35 and 7.6[15] or $K_a > 10^{10}$ M^{-1}.

The basics of the self-cleavage technique are that the complex between the restriction enzyme and specific DNA fragment preformed 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 vs the specific fragment at a 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 a 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 min at 20 °C; the cleavage reaction was then stopped by adding EDTA to a final concentration of 20 mM. As demonstrated previously[15] under these experimental conditions, the fraction of DNA cleaved reflects the fraction of DNA initially bound to EcoRV.

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

Gel Electrophoresis. Loading dye was added to the purified DNA digestion products to final concentrations 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, and 0.5 mM EDTA (pH 8.3)]. Samples were allowed to run at 350 V for 2.5−3 h. The reaction products of supercoiled plasmid cleavage were loaded directly onto 1.3% agarose gels in TAE and run at 50 V for 18 h.

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 instrument was interfaced with a Pentium personal computer. Band intensities were quantified using the Fuji Film software MultiGauge for Windows. Intensity data generated in MultiGauge were further analyzed using SigmaPlot version 10.0 (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 the initiation of cleavage with $MgCl_2$, the preformed DNA−protein complex (ES) can either proceed to intermediate ES* with rate constant k_1 or dissociate to E and S with rate constant k_d. The intermediate can then proceed to double-stranded cleavage product P with rate constant k_2. The kinetic

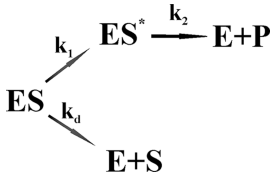

Figure 1. Minimal model for Mg^{2+} binding in the EcoRV cleavage reaction that satisfies the observed kinetics. Starting from the specifically bound conformation (ES), EcoRV can proceed to an intermediate form (ES*) with rate constant k_1 or the complex can dissociate from the DNA (E + S) with rate 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.

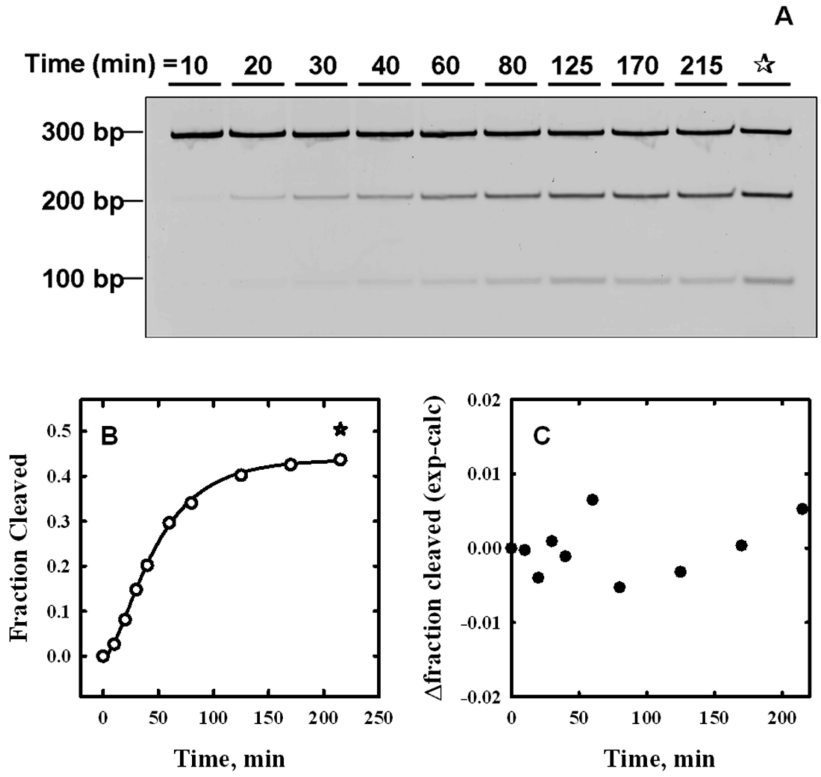

Figure 2. Single-turnover experiments were performed to measure EcoRV cleavage kinetics. (A) A gel image illustrating the results of the cleavage kinetics experiment is shown. Cleavage of the 310 bp fragment gives the two fragments that are ~100 and ~200 bp in length. Fractions of cleaved DNA fragment 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). Reaction mixtures were incubated at 20 °C. The oligonucleotide is added to trap any free enzyme. The star designates an experimental control for the fraction of DNA bound by EcoRV in the initial DNA–protein pool that was preincubated in the absence of Mg^{2+}. The fraction of DNA cleaved for the control point was obtained under the same conditions described above except that 10 mM $MgCl_2$ and imidazole buffer (pH 7.0) were used in the final reaction mixture. The control sample was incubated with the cleavage mix for 10 min. Under these conditions, the fraction of DNA cleaved reflects the fraction of DNA initially bound. (B) Time dependence of the fraction of DNA cleaved by EcoRV calculated from the gel displayed in panel A. 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 eq 1) with the following parameters: k_1 = 0.064 min^{-1}, k_2 = 0.025 min^{-1}, and $k_d = 9.9 \times 10^{-3}$ min^{-1}. (C) Differences in the fraction cleaved for the experimental points and the theoretical curve at each time point reveal differences of <2% between the values, confirming that the minimal model provides an adequate fit to the experimental data.

equations associated with this scheme can be straightforwardly solved for the fraction cleaved to give

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

where $[ES]_0$ is the initial concentration of the 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 the 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 from Parametric Technology Corp.

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, $\log(a) = -$pH, and for water, $\ln(a) = -$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 a divalent cofactor with a 310 bp DNA fragment containing a single EcoRV-specific site for

dx.doi.org/10.1021/bi401089y | Biochemistry 2014, 53, 235–246

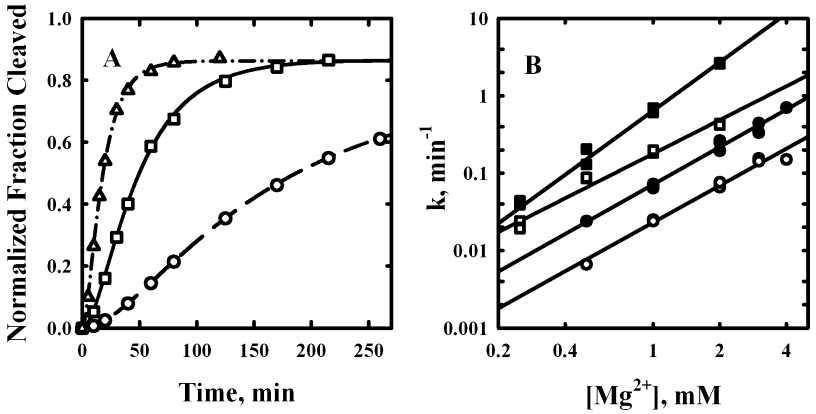

Figure 3. Cleavage kinetics and Mg^{2+} concentration. (A) The rates of single-turnover DNA cleavage were measured at 0.5 (○), 1.0 (□), and 2.0 (△) mM $MgCl_2$, 3.0 osmolal glycine betaine, 100 mM NaCl, and 20 mM imidazole buffer (pH 6.35). Lines correspond to the best fits obtained using eq 1. (B) Parameters k_1 and k_2, derived from the set of experiments analogous to those shown in panel A at pH 6.35 [(●) k_1 and (○) k_2] and pH 6.88 [(■) k_1 and (□) 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 the number of Mg^{2+} ions associated with the particular kinetic step using eq 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 like those outlined in the legend of Figure 2.

sufficient time to ensure that all protein is specifically bound to the DNA fragment.[15] Typically, the enzyme: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. An increased level of cleavage is apparent with increased incubation times with Mg^{2+}. Figure 2B shows the time dependence of cleavage. The star in Figure 2A shows the fraction of initially bound protein. The initial fraction of the specific complex is determined by a standard self-cleavage assay: the final reaction mix contains 10 mM $MgCl_2$, a pH value of ∼7 adjusted with imidazole, and 3 osmolal glycine betaine. Under these conditions, virtually all initially protein-bound DNA fragments are cleaved after incubation for 10 min; complex dissociation during this time is negligible.[15,28]

The fraction of DNA cleaved (Figure 2B) reaches a plateau only after incubation of the DNA−protein complex in the cleavage reaction mixture for 180 min; the presence of a plateau is consistent with the expected behavior for a single-turnover reaction. Two prominent features are apparent in the kinetic 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 the fraction DNA fragment cleaved is ∼14% lower than the fraction that corresponds to the initial fraction of the prebound fragment (star in Figure 2A,B), indicating that there is a weak but measurable dissociation of the prebound protein from the fragment without double-stranded cleavage during the time course of the experiment.

The formation of the product, i.e., the fraction of DNA cleaved, can be straightforwardly calculated using eq 1, derived for the minimal reaction scheme shown in Figure 1. The solid line in Figure 2B shows the best fit of eq 1 to the data. Figure 2C shows the fit residuals. The average difference in the 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 conducted in at least 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. Because dissociation was minimized, the error in k_d was significantly greater but did not significantly affect k_1 or k_2.

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 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 eq 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 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 osmolal glycine betaine and at pH 6.88 with 4 osmolal 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

dx.doi.org/10.1021/bi401089y | Biochemistry 2014, 53, 235−246

are taken up at pH 6.35 and 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 shown 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 Supporting Information shows that the data for k_1 at pH 6.88, for example, can clearly distinguish among $\Delta N^{\dagger}_{Mg^{2+}}$ values of 1, 2, and 3.

Table 1. Numbers of Mg^{2+} Ions ($\Delta N^{\dagger}_{Mg^{2+}}$) Associated with Each Kinetic Step of the EcoRV Cleavage Reaction for Three Different pH and Osmotic Pressure Values

		$\Delta N^{\dagger}_{Mg^{2+}}$		
pH	betaine osmotic pressure (osmolal)	k_1	k_2	total
6.35	3	1.6 ± 0.1	1.6 ± 0.1	3.2 ± 0.2
6.88	4	2.0 ± 0.06	1.4 ± 0.09	3.4 ± 0.15
7.40[a]	5	2.4 ± 0.2	1.3 ± 0.3	3.7 ± 0.5

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

Cleavage Kinetics and pH. EcoRV cleavage kinetic curves were measured at different pH values ranging from 6.35 to 7.4 while the Mg^{2+} and glycine betaine concentrations were kept constant. Figure 4A shows two sets of experimental data and corresponding fits for pH 6.35 (●) and pH 6.88 (○). Both curves were measured at 2 mM $MgCl_2$ and 4 osmolal betaine glycine. Under these conditions, the extent of enzyme dissociation is <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 concentrations of 0.5 mM Mg^{2+} and 3 osmolal glycine betaine. The pH dependence of the ratio of nonspecific and specific equilibrium constants, K_{nsp-sp}, determined previously[15] is also shown for comparison. The curves are reasonably linear over the pH range of ~6.3−6.9 for $\log(k_1)$ and $\log(k_2)$ and ~5.5−6.5 for $\log(K_{nsp-sp})$. The slope can be related to a difference in the 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 dependence of $\log(K_{nsp-sp})$ on pH is 2.0 ± 0.3, indicating that approximately two fewer H^+ ions are bound to the specific complex than 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 approximately four H^+ ions are released by the protein as the complement of three to four Mg^{2+} ions are bound. Table 2 indicates that $\Delta N^{\dagger}_{H^+}$ is weakly dependent on Mg^{2+} concentration and osmotic pressure.

Table 2. Numbers of Protons ($\Delta N^{\dagger}_{H^+}$) Associated with Each Kinetic Step of the EcoRV Cleavage Reaction for Three Different Osmotic Pressures and Mg^{2+} Concentrations

		$\Delta N^{\dagger}_{H^+}$		
betaine osmotic pressure (osmolal)	$[Mg^{2+}]$ (mM)	k_1	k_2	total
3	0.5	2.3 ± 0.3	2.2 ± 0.4	4.5 ± 0.7
4	2.0	2.4 ± 0.08	1.8 ± 0.05	4.2 ± 0.13
3	4.0[a]	2.6	1.9	4.5

[a]Only two points were measured; MES buffer was used at pH 6.0 and imidazole at pH 6.35.

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.

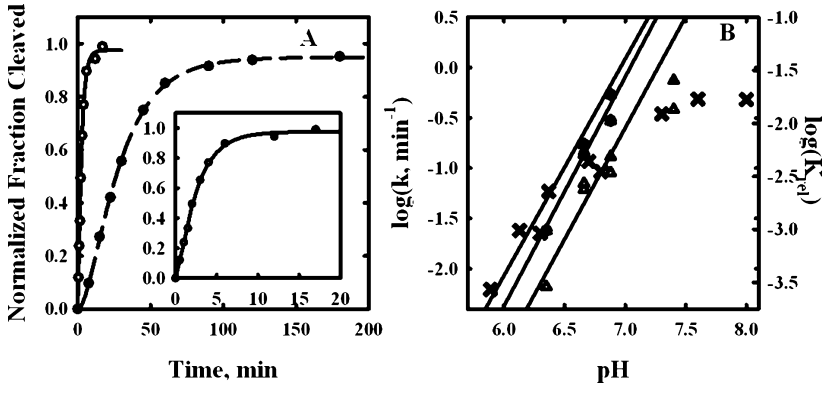

Figure 4. Cleavage kinetics and pH. (A) Single-turnover cleavage experiments were performed at pH 6.35 (●) and pH 6.88 (○) in 2 mM $MgCl_2$ and 4 osmolal glycine betaine. The lines correspond to the best theoretical fits obtained using eq 1. The inset shows an expanded view of the kinetics at pH 6.88.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

dx.doi.org/10.1021/bi401089y | Biochemistry 2014, 53, 235−246

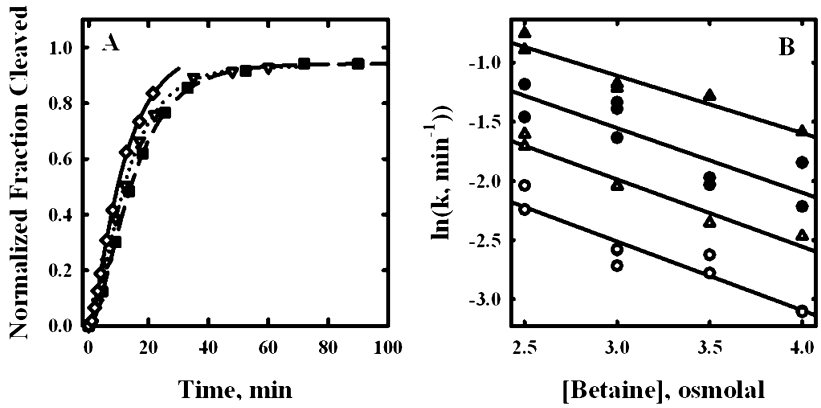

Figure 5. Cleavage kinetics and osmotic pressure. (A) Kinetic curves for three experiments performed at pH 6.88, 0.5 mM $MgCl_2$, and osmotic pressures of 3 (◇), 3.5 (▽), and 4 (■) osmolal glycine betaine. At 3 osmolal glycine betaine, k_1 = 0.5382 min^{-1} and k_2 = 0.0902 min^{-1}, whereas at 4 osmolal betaine, k_1 = 0.2046 min^{-1} and k_2 = 0.0851 min^{-1}. (B) Rates from the curves analogous to those shown in panel A measured at pH 6.35 [(●) k_1 and (○) k_2] and 6.88 [(▲) k_1 and (△) 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), respectively. Other conditions were like those described in the legend of Figure 2.

$$\frac{d[\ln(k)]}{d[\text{osmolal}]} = -\frac{\Delta N_w^{\dagger}}{55.5} \quad (5)$$

where $\Delta N_w^{\dagger}$ 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, a constant $MgCl_2$ concentration of 0.5 mM, and pH 6.88 and their corresponding fits are shown in Figure 5A. The kinetic rate constants k_1 and k_2 decrease with an increase in 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 eq 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 shown 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 s^{-1}. This is <2-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).

Table 3. Numbers of Water Molecules ($\Delta N_w^{\dagger}$) Associated with Each Kinetic Step of the EcoRV Cleavage Reaction for Three Different Mg^{2+} Concentrations and pH Values

		$\Delta N_w^{\dagger}$		
pH	[Mg^{2+}] (mM)	k_1	k_2	total
6.35	2	30 ± 7	32 ± 5	62 ± 12
6.88	0.5	27 ± 8	32 ± 6	59 ± 14
7.40[a]	0.5	35 ± 9	36 ± 3	72 ± 12

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

Mg^{2+} Binding Is Coupled to Single-Strand Cleavage. Halford and co-workers[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 the second linearizes it. The three species can be separated on agarose gels. We have repeated these experiments using osmotic stress 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 eq 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 pH 6.35 and 7.4 (data not shown), the k_1 rate constants are approximately 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 the 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. Approximately 40 water molecules are seen linked to each kinetic step, ~10 more than the number seen for the DNA fragment at pH 6.35. Mg^{2+} binding and single-strand cleavage are tightly coupled.

dx.doi.org/10.1021/bi401089y | *Biochemistry* 2014, 53, 235−246

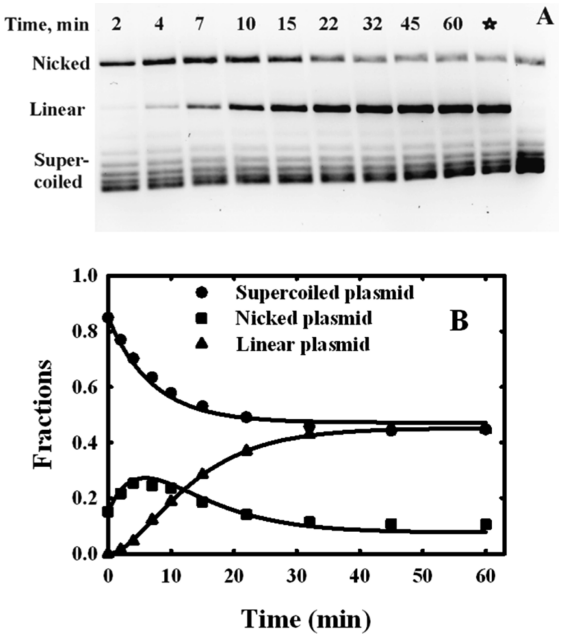

Figure 6. Cleavage kinetics of the supercoiled plasmid. (A) Gel image illustrating the cleavage kinetics of the supercoiled plasmid. Plasmid Litmus28i was preincubated with EcoRV in 100 mM NaCl, pH 6.35, and 3 osmolal glycine betaine at 20 °C in the absence of Mg^{2+}. The cleavage reaction was initiated 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 conditions of 100 mM NaCl, pH 6.35, and 3 osmolal glycine betaine. At various times, aliquots were taken and the reaction was 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 star was the control experiment for total enzyme initially bound to plasmid. The last lane on the right contained untreated plasmid. The bands for nicked, relaxed circle, linear plasmid, and supercoiled DNA are labeled. Note the initial increase and subsequent decrease in the intensity of the relaxed circle band. (B) Relative fractions of nicked (■), linear (▲), and supercoiled (●) DNA determined from staining intensities shown as a function of time. The linear DNA data were fit with eq 1 and are 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.

■ DISCUSSION

Because previous cleavage kinetic experiments[12,14,32] have yielded 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 of 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 are 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 to seconds.[14,30,33] By using osmotic pressure, however, the cleavage kinetics of EcoRV are slowed enough to allow 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 nonperturbing solutes used *in vivo* to protect cells from high osmotic stress. This compatible osmolyte is found in a wide variety of bacteria, plants, and marine animals and in the mammalian renal medulla.[34,35] DNA−protein complexes can withstand extremely high concentrations of glycine betaine without being damaged. 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 of 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 2 of the equivalent rate constant measured by Baldwin et al.[14] under similar experimental conditions. This is a further strong indication that glycine betaine, even at a quite high osmolality, is only acting osmotically. An additional utility of using glycine betaine as an osmolyte is that it does not significantly change the activity of either Na^+ or Mg^{2+} ions up to 5 osmolal glycine betaine [confirmed using ion selective electrode (data not shown)]. The p*K* 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 incubation of the DNA−protein complex with the cleavage mix for ∼180 min. The presence of the plateau indicates that only the DNA fragments that were prebound by protein were cleaved throughout the duration of the experiment. The plateau value of the cleaved fraction is somewhat smaller than the fraction of the 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 occurs only after the second step.

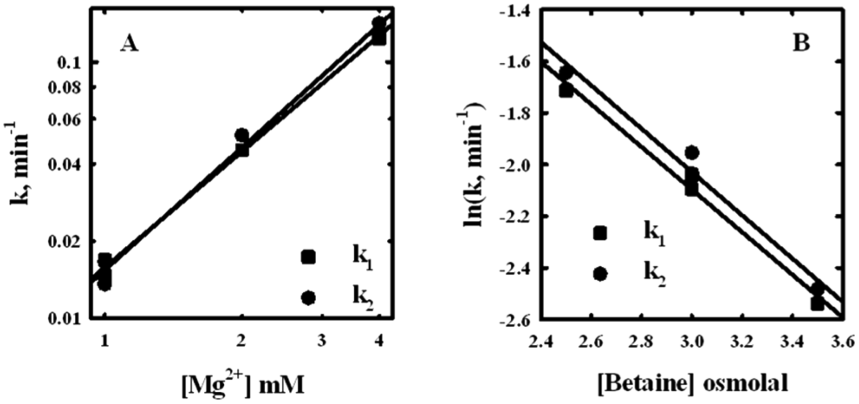

Figure 7. Dependence of supercoil cleavage kinetics on Mg^{2+} concentration and osmotic pressure. (A) Dependence of k_1 and k_2 on Mg^{2+} concentration for pH 6.35 and 3 osmolal betaine at 20 °C shown as a log−log plot as in Figure 3B. For both rate steps, $\Delta N^{\dagger}_{Mg^{2+}} = 1.6 \pm 0.4$ Mg^{2+} 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) Dependence of $\ln(k)$ on glycine betaine osmolality shown for k_1 and k_2 as in Figure 5B. For both reaction steps, $\Delta N^{\dagger}_{w} = 42 \pm 6$ water molecules, ~10 more than the number observed for the DNA fragment.

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 eq 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 because 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. To characterize the physical processes underlying rates k_1 and k_2, we measured the 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 (Supporting Information). The lag phase observed by Baldwin et al. incorporates the kinetics of dissociation, binding of Mg^{2+} to free enzyme, and 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 the final cleavage step without dissociation. The plots shown in Figure 3B are all quite linear, indicating that the number of Mg^{2+} ions bound per enzyme coupled to k_1 and k_2 is constant over the Mg^{2+} concentration range measured, 0.25−2 mM for pH 6.88 and 0.5−4 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 four 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 an increase in 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 one and two Mg^{2+} ions 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 because of 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+}, and 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.

The EcoRV enzymatic cleavage activity decreases dramatically as the 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 level of 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 the 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 pK_a values of these Glu and Asp groups seem to be considerably shifted because of 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 pK_a values between the specific and nonspecific complexes. The pH dependence of the single-turnover cleavage kinetics in the range of 6.35−6.9 with constant concentrations of 0.5 mM Mg^{2+} and 3 osmolal glycine betaine showed that both 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 approximately four 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 Mg^{2+} would be expected to shift the pK_a of the Asp and Glu residues in the active site to more normal values, resulting in the release

dx.doi.org/10.1021/bi401089y | Biochemistry 2014, 53, 235−246

of approximately two H^+ ions per dimer for pH values in the range of 6.5. Our previous equilibrium competitive binding experiments in the absence of Mg^{2+} indicated that approximately two more H^+ ions are bound by the specific complex than by 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 of 6.35−6.9. The Mg^{2+} binding constant of the specific complex decreases with a decrease in pH. The pH dependences of k_1, k_2, and K_{rel} are much smaller for pH values greater than ∼7, indicating that at higher pH values 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 five 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 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] cannot be readily accessed by 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, ∼4−5-fold greater than the number 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. Because 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 the 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 co-workers[30,31] results from the binding of the first set of Mg^{2+} ions. In contrast, however, the binding of a complement of two Mg^{2+} ions to one enzyme monomer of the specific complex in the crystal[8] did not result in cleavage. Given the binding of ∼30 extra water molecules coupled to binding of Mg^{2+} 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 k_1/k_2 ratio 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 the number of Mg^{2+} ions coupled to the k_1 step with an increase in 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.

The cellular concentration of free Mg^{2+} has been estimated to be in the range of 0.5−4 mM,[45,46] significantly lower than the $MgCl_2$ range of 10−30 mM 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 four 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 the free enzyme with a full complement of four Mg^{2+} ions can bind and cleave DNA efficiently, i.e., that the enzyme without a full Mg^{2+} complement can bind only nonproductively or bind and cleave DNA at a much lower rate. Alternatively, the 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 constant for binding of Mg^{2+} to the complex is much larger than that for binding to the free enzyme. The binding constant for association of EcoRV with bound divalent ions to its DNA recognition site is estimated as at least ∼100-fold larger than that for the metal-free enzyme.[37] This also means that binding of a divalent ion 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 approximately four Mg^{2+} ions per dimer are bound by the enzyme at pH 7.4 even at 0.25 mM Mg^{2+}. The two Mg^{2+} ions per monomer requirement for cleavage has been experimentally verified. In the pH range of 6.35−6.9, approximately four H^+ protons per dimer are released in total, likely because of titrations of aspartate or glutamate residues in the catalytic sites and the formation of Mg^{2+}-activated hydroxides that cleave or 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.

■ ASSOCIATED CONTENT

Supporting Information

Predicted dependence of rate constants on Mg^{2+} concentration assuming ΔN = 1, 2, and 3, demonstrating that the data discriminate well among these values and a demonstration that, 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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: raud@mail.nih.gov. Phone: (301) 402-4698.

Funding

This work was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We greatly appreciate the generous gift of *Escherichia coli* strain CSH50 [pMetB] and plasmid mix pBSKSRVD/pMetB from Dr. Andrea Prota and Dr. Fritz Winkler (Paul Scherrer Institut, Villigen, Switzerland). We are deeply grateful to Dr. Galina Obmolova (Centocor) and Dr. Andrea Prota for their valuable advice regarding EcoRV purification.

■ ABBREVIATIONS

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

■ REFERENCES

(1) Cowan, J. A. (1998) Metal Activation of Enzymes in Nucleic Acid Biochemistry. *Chem. Rev. 98*, 1067−1088.
(2) Dupureur, C. M. (2008) Roles of metal ions in nucleases. *Curr. Opin. Chem. Biol. 12*, 250−255.
(3) Dupureur, C. M. (2010) One is enough: Insights into the two-metal ion nuclease mechanism from global analysis and computational studies. *Metallomics 2*, 609−620.
(4) Pingoud, A., Fuxreiter, M., Pingoud, V., and Wende, W. (2005) Type II restriction endonucleases: Structure and mechanism. *Cell. Mol. Life Sci. 62*, 685−707.
(5) Pingoud, V., Wende, W., Friedhoff, P., Reuter, M., Alves, J., Jeltsch, A., Mones, L., Fuxreiter, M., and Pingoud, A. (2009) On the divalent metal ion dependence of DNA cleavage by restriction endonucleases of the EcoRI family. *J. Mol. Biol. 393*, 140−160.
(6) Horton, N. C., Newberry, K. J., and Perona, J. J. (1998) Metal ion-mediated substrate-assisted catalysis in type II restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A. 95*, 13489−13494.
(7) Horton, N. C., and Perona, J. J. (2004) DNA cleavage by EcoRV endonuclease: Two metal ions in three metal ion binding sites. *Biochemistry 43*, 6841−6857.
(8) Kostrewa, D., and Winkler, F. K. (1995) Mg^{2+} binding to the active site of EcoRV endonuclease: A crystallographic study of complexes with substrate and product DNA at 2 Å resolution. *Biochemistry 34*, 683−696.
(9) Horton, N. C., Connolly, B. A., and Perona John, J. (2000) Inhibition of EcoRV endonucleaase by deoxyribo-3′-S-phosphorothiolates: A high-resolution X-ray crystallographic study. *J. Am. Chem. Soc. 122*, 3314−3324.
(10) Vipond, I. B., Baldwin, G. S., and Halford, S. E. (1995) Divalent metal ions at the active sites of the EcoRV and EcoRI restriction endonucleases. *Biochemistry 34*, 697−704.
(11) Xie, F., Qureshi, S. H., Papadakos, G. A., and Dupureur, C. M. (2008) One- and two-metal ion catalysis: Global single-turnover kinetic analysis of the PvuII endonuclease mechanism. *Biochemistry 47*, 12540−12550.
(12) Groll, D. H., Jeltsch, A., Selent, U., and Pingoud, A. (1997) Does the restriction endonuclease EcoRV employ a two-metal-ion mechanism for DNA cleavage? *Biochemistry 36*, 11389−11401.
(13) Zahran, M., Berezniak, T., Imhof, P., and Smith, J. C. (2011) Role of magnesium ions in DNA recognition by the EcoRV restriction endonuclease. *FEBS Lett. 585*, 2739−2743.
(14) Baldwin, G. S., Vipond, I. B., and Halford, S. E. (1995) Rapid reaction analysis of the catalytic cycle of the EcoRV restriction endonuclease. *Biochemistry 34*, 705−714.
(15) Sidorova, N. Y., Muradymov, S., and Rau, D. C. (2011) Solution parameters modulating DNA binding specificity of the restriction endonuclease EcoRV. *FEBS J. 278*, 2713−2727.
(16) Garner, M. M., and Rau, D. C. (1995) Water release associated with specific binding of gal repressor. *EMBO J. 14*, 1257−1263.
(17) Fried, M. G., Stickle, D. F., Smirnakis, K. V., Adams, C., MacDonald, D., and Lu, P. (2002) Role of hydration in the binding of lac repressor to DNA. *J. Biol. Chem. 277*, 50676−50682.
(18) Poon, J., Bailey, M., Winzor, D. J., Davidson, B. E., and Sawyer, W. H. (1997) Effects of molecular crowding on the interaction between DNA and the *Escherichia coli* regulatory protein TyrR. *Biophys. J. 73*, 3257−3264.
(19) Rau, D. C. (2006) Sequestered water and binding energy are coupled in complexes of lambda Cro repressor with non-consensus binding sequences. *J. Mol. Biol. 361*, 352−361.
(20) Vossen, K. M., Wolz, R., Daugherty, M. A., and Fried, M. G. (1997) Role of macromolecular hydration in the binding of the *Escherichia coli* cyclic AMP receptor to DNA. *Biochemistry 36*, 11640−11647.
(21) Robinson, C. R., and Sligar, S. G. (1996) Participation of water in Hin recombinase−DNA recognition. *Protein Sci. 5*, 2119−2124.
(22) Li, L., and Matthews, K. S. (1997) Differences in water release with DNA binding by ultrabithorax and deformed homeodomains. *Biochemistry 36*, 7003−7011.
(23) Robinson, C. R., and Sligar, S. G. (1998) Changes in solvation during DNA binding and cleavage are critical to altered specificity of the EcoRI endonuclease. *Proc. Natl. Acad. Sci. U.S.A. 95*, 2186−2191.
(24) Sidorova, N. Y., and Rau, D. C. (1996) Differences in water release for the binding of EcoRI to specific and nonspecific DNA sequences. *Proc. Natl. Acad. Sci. U.S.A. 93*, 12272−12277.
(25) Sidorova, N. Y., and Rau, D. C. (2001) Linkage of EcoRI dissociation from its specific DNA recognition site to water activity, salt concentration, and pH: Separating their roles in specific and non-specific binding. *J. Mol. Biol. 310*, 801−816.
(26) Sidorova, N. Y., and Rau, D. C. (2004) Differences between EcoRI nonspecific and "star" sequence complexes revealed by osmotic stress. *Biophys. J. 87*, 2564−2576.
(27) Sidorova, N. Y., Muradymov, S., and Rau, D. C. (2006) Differences in hydration coupled to specific and nonspecific competitive binding and to specific DNA binding of the restriction endonuclease BamHI. *J. Biol. Chem. 281*, 35656−35666.
(28) Sidorova, N. Y., Muradymov, S., and Rau, D. C. (2005) Trapping DNA-protein binding reactions with neutral osmolytes for the analysis by gel mobility shift and self-cleavage assays. *Nucleic Acids Res. 33*, 5145−5155.
(29) Luke, P. A., McCallum, S. A., and Halford, S. E. (1987) The EcoR V restriction endonuclease. *Gene Amplif. Anal. 5*, 185−207.
(30) Erskine, S. G., Baldwin, G. S., and Halford, S. E. (1997) Rapid-reaction analysis of plasmid DNA cleavage by the EcoRV restriction endonuclease. *Biochemistry 36*, 7567−7576.
(31) Halford, S. E., and Goodall, A. J. (1988) Modes of DNA cleavage by the EcoRV restriction endonuclease. *Biochemistry 27*, 1771−1777.

(32) Jeltsch, A., Alves, J., Wolfes, H., Maass, G., and Pingoud, A. (1993) Substrate-assisted catalysis in the cleavage of DNA by the EcoRI and EcoRV restriction enzymes. *Proc. Natl. Acad. Sci. U.S.A. 90*, 8499−8503.
(33) Stanford, N. P., Halford, S. E., and Baldwin, G. S. (1999) DNA cleavage by the EcoRV restriction endonuclease: pH dependence and proton transfers in catalysis. *J. Mol. Biol. 288*, 105−116.
(34) Yancey, P. H. (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol. 208*, 2819−2830.
(35) Nakanishi, T., Turner, R. J., and Burg, M. B. (1990) Osmoregulation of betaine transport in mammalian renal medullary cells. *Am. J. Physiol. 258*, F1061−F1067.
(36) Engler, L. E., Welch, K. K., and Jen-Jacobson, L. (1997) Specific binding by EcoRV endonuclease to its DNA recognition site GATATC. *J. Mol. Biol. 269*, 82−101.
(37) Martin, A. M., Horton, N. C., Lusetti, S., Reich, N. O., and Perona, J. J. (1999) Divalent metal dependence of site-specific DNA binding by EcoRV endonuclease. *Biochemistry 38*, 8430−8439.
(38) Pingoud, A., and Jeltsch, A. (2001) Structure and function of type II restriction endonucleases. *Nucleic Acids Res. 29*, 3705−3727.
(39) Haq, I., O'Brien, R., Lagunavicius, A., Siksnys, V., and Ladbury, J. E. (2001) Specific DNA recognition by the type II restriction endonuclease MunI: The effect of pH. *Biochemistry 40*, 14960−14967.
(40) Reid, S. L., Parry, D., Liu, H. H., and Connolly, B. A. (2001) Binding and recognition of GATATC target sequences by the EcoRV restriction endonuclease: A study using fluorescent oligonucleotides and fluorescence polarization. *Biochemistry 40*, 2484−2494.
(41) Winkler, F. K., and Prota, A. E. (2003) Structure and Function of EcoRV Endonuclease. In *Restriction Endonucleases* (Pingoud, A., Ed.) pp 179−214, Springer-Verlag, Berlin.
(42) Parsegian, V. A., Rand, R. P., and Rau, D. C. (1995) Macromolecules and water: Probing with osmotic stress. *Methods Enzymol. 259*, 43−94.
(43) Parsegian, V. A., Rand, R. P., and Rau, D. C. (2000) Osmotic stress, crowding, preferential hydration, and binding: A comparison of perspectives. *Proc. Natl. Acad. Sci. U.S.A. 97*, 3987−3992.
(44) Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K., and Wilson, K. S. (1993) The crystal structure of EcoRV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J. 12*, 1781−1795.
(45) Lusk, J. E., Williams, R. J., and Kennedy, E. P. (1968) Magnesium and the growth of *Escherichia coli*. *J. Biol. Chem. 243*, 2618−2624.
(46) Cowan, J. A. (1995) Introduction to the Biological Chemistry of Magnesium Ion. In *The Biological Chemistry of Magnesium* (Cowan, J. A., Ed.) pp 1−23, Wiley-VCH, New York.