ABSTRACT
We take advantage of our previous observation that neutral osmolytes can strongly slow down the rate of DNA–protein complex dissociation to develop a method that uses osmotic stress to ‘freeze’ mixtures of DNA–protein complexes and prevent further reaction enabling analysis of the products. We apply this approach to the gel mobility shift assay and use it to modify a self-cleavage assay that uses the nuclease activity of the restriction endonucleases to measure sensitively their specific binding to DNA. At sufficiently high concentrations of neutral osmolytes the cleavage reaction can be triggered at only those DNA fragments with initially bound enzyme. The self-cleavage assay allows measurement of binding equilibrium and kinetics directly in solution avoiding the intrinsic problems of gel mobility shift and filter binding assays while providing the same sensitivity level. Here we compare the self-cleavage and gel mobility shift assays applied to the DNA binding of EcoRI and BamHI restriction endonucleases. Initial results indicate that BamHI dissociation from its specific DNA sequence is strongly linked to water activity with the half-life time of the specific complex increasing ~20-fold from 0 to 1 osmolal betaine.

INTRODUCTION
The equilibrium binding of sequence specific DNA binding proteins to their recognition sequences can be analyzed by methods based on physical separation of complexes from free DNA fragments, as, e.g. with the gel mobility shift or the filter binding assays or by the techniques probing DNA–protein equilibrium directly in a solution, e.g. fluorescence and calorimetry. These latter techniques require larger amounts of materials and do not typically provide enough sensitivity for measuring equilibrium association constants in the range $10^{-10}$–$10^{-12}$ M$^{-1}$. Both gel mobility shift and filter binding assays can measure these large constants, but the process of physically separating DNA–protein complex from free species can also perturb the equilibrium. Indeed, solution studies and gel mobility shift/filter binding assays do sometimes produce quite different results. Additionally, gel mobility shift assay can also demonstrate frustratingly different results for the same DNA–protein system but using different electrophoresis conditions. Controversy still exists in the literature, e.g. regarding the ability of the EcoRV restriction endonuclease to bind DNA specifically in the absence of divalent metal ions. Reports from groups using gel mobility shift and/or filter binding assays vary from no observed specificity (1–3) to ~1000-fold preference for binding of EcoRV to the specific sequence over nonspecific DNA (4). Subsequent fluorescence studies indicated that in the absence of divalent metal ion EcoRV is either unable to distinguish its specific sequence from nonspecific DNA sites (5), or has a weak (1.5–6.5) preference for specific versus nonspecific DNA sequences (6).

How quickly the initial equilibrium can be perturbed by separation techniques depends on the association and dissociation rates of the DNA–protein complex. Typically, competitor DNA is added to prevent the association of free protein with the DNA of interest. Dissociation rates are more difficult to control. We have seen that the specific complex dissociation rates for two DNA binding proteins, EcoRI restriction nuclease (7,8) and $\lambda$ Cro repressor (unpublished data), decrease strongly with increasing concentrations of neutral osmolytes. This sensitivity arises from the net uptake of many waters by the complex during the dissociation reaction; the mainly direct protein–DNA interactions that occur in the specific complex are replaced by waters of hydration. The free energy change from binding this extra water is naturally linked to the water activity or osmolyte concentration of the bulk solution.

Osmolytes can be added to high concentration without apparently affecting the integrity of the complex. We have
developed a method that uses the osmotic stress approach to ‘freeze’ mixtures of DNA–protein complexes and prevent further reaction for analysis by the gel mobility shift assay. Such a ‘stop reaction’ must necessarily block dissociation of already formed complex while preventing the association of free protein with the target DNA. We find that adding high concentrations of neutral osmolytes and an excess of the specific sequence oligonucleotide competency satisfies these conditions allowing the gel mobility shift assay to measure accurately the equilibrium distribution of free and bound species in solution before ‘freezing’ the reaction.

We have also developed and report here a novel variant of the self-cleave assay that uses the nucleic activity of restriction endonucleases to measure sensitively their specific binding. At sufficiently high concentrations of neutral osmolytes (such as betaine glycine, triethylene glycol or methyl glucoside) and sequence specific oligonucleotide the cleavage reaction is only triggered at those DNA fragments already with bound enzyme. Under these conditions the fraction of specific DNA fragment cleaved reflects the fraction of DNA bound to the enzyme before initiating the cleavage reaction.

We compare here the self-cleave and gel mobility shift assays applied to the dissociation kinetics and binding equilibrium of two restriction endonucleases, EcoRI and BamHI. Using self-cleave assay we demonstrate that BamHI dissociation from its specific DNA sequence is strongly linked to water activity with half-life time of the specific complex increasing ~20-fold from 0 to 1 osmol betaine.

**MATERIALS AND METHODS**

**Materials**

A 360 bp DNA fragment containing a single EcoRI recognition sequence was isolated from a PvuII digestion of a modified pUC19 plasmid using standard techniques. A 349 bp fragment containing a single BamHI specific site was purified from a PvuII digestion of pNEB193 plasmid. Plasmid DNA and restriction enzyme PvuII were purchased from New England Biolabs.

The double-stranded 30 bp long oligonucleotides used in experiments were: EcoRI-specific sequence oligo: ACGACGGCCAGTGAATTGCAGCTCGGTACC; BamHI specific sequence oligo: CCAGAAGTCCCGGATCCCTTAACCGAGTC; BamHI nonspecific sequence oligo: CCGGAGACTCCCGGATCCCTTAACCGAGTC. EcoRI and BamHI specific sequences are shown in bold letters. The BamHI nonspecific sequence oligonucleotide contains the inverted BamHI recognition sequence (underlined) in place of the specific recognition sequence.

The oligonucleotides shown above and their complements were purchased from Invitrogen Inc. and dissolved in STE buffer [100 mM NaCl, 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA]. Complementary strands were mixed in 1:1 proportion, heated to 92 °C, and annealed by slow cooling to 25 °C. Small molecular mass impurities were removed using P6 Bio-Spin columns at room temperature. Double-stranded oligonucleotides were then ethanol precipitated, and dissolved in TE buffer [10 mM Tris–HCl (pH 7.5) and 1 mM EDTA]. The purity of the double-stranded oligonucleotides was confirmed by PAGE. The concentrations of the DNA fragment and oligonucleotides were determined spectrophotometrically, using an extinction coefficient of 0.013 (μM base pairs)⁻¹ at 260 nm. Absorption spectra were obtained with a Perkin Elmer Lambda 800 UV-Vis spectrophotometer.

DNA binding and cleavage experiments were performed with highly purified EcoRI restriction endonuclease (a kind gift of Dr L. Jen-Jacobson) and with highly purified BamHI restriction endonuclease (a kind gift of Dr A. Aggarwal). Active protein concentrations of the EcoRI and BamHI were determined by direct titration with the 360 and 349 bp DNA fragments containing corresponding specific recognition sequences under conditions of stoichiometric binding as described previously (9,10).

Betaine glycine was purchased from The United States Biochemical, α-methyl glucoside and triethylene glycol from Sigma Chemical Co. All solutes were used without further purification. Osmotol concentrations of solutes were determined by direct measurement using a vapor pressure osmometer operating at room temperature (Wescor, Logan, UT; model 5520×R). Changes in water chemical potentials are linearly proportional to solute osmotic concentrations, i.e., \( \Delta \mu_w = \mu_w - \mu_w^{ref} = -RT [\text{osmolar}] / 55.6 \), where \( \mu_w \) and \( \mu_w^{ref} \) are the water chemical potentials of the solutions with and without added osmolyte.

**Equilibrium binding experiments**

In DNA-BamHI binding experiments samples contained 20 mM imidazole (pH 7.0), 60 or 100 mM KCl, 2 mM DTT, 0.05 mg/ml acetylated BSA and 0.1 or 2 mM EDTA. DNA-EcoRI samples contained 20 mM imidazole (pH 7.0 or 7.5), 75 or 90 mM NaCl, 2 mM DTT, 0.05 mg/ml acetylated BSA and 0.1 or 2 mM EDTA. The total reaction volume was 30 μl.

Specific and nonspecific equilibrium competition experiments were performed as described previously (7,9,10). Briefly, mixtures of BamHI (~1.5 nM), the specific site fragment (~3 nM) and the nonspecific oligonucleotide competitor (between 0 and ~50 μM in oligonucleotide or 0–~1.5 mM in bp), were incubated at 20 °C for 2.5 h, a time long enough to reach equilibrium as indicated by measurements of specific complex dissociation rates (see Results). The loss of specific site binding as the concentration of nonspecific competitor DNA increased was determined by both the gel mobility shift and the self-cleave assays (self-cleave assay is described in detail in the Results section).

To prevent re-equilibration of samples in the electrophoretic well before entering the gel, a stop reaction mixture was added to ‘freeze’ the equilibrium fraction of specifically bound protein. Before loading samples on a gel, 30 μl of the stop reaction mixture was added to each sample. The final solution after adding the stop reaction mixture contained 200-fold molar excess of the specific sequence oligonucleotide versus specific fragment and 2 osmolal betaine glycine. In case of the DNA–BamHI complexes, the stop reaction mixture also contained 2 mM CaCl₂ to ensure 2 mM Ca²⁺ in the final samples. The increased osmotic pressure (and presence of Ca²⁺ in case of BamHI) lengthens the dissociation half-life time of the EcoRI and BamHI specific complexes to at least several hours (7,10,11) and (Figure 9). In the absence of Mg²⁺ we observed
no measurable cleavage of the DNA in the presence or absence of Ca$^{2+}$.

**Dissociation kinetics**

Solution conditions for BamHI kinetic experiments were 20 mM imidazole pH 7.0 and 60 mM KCl. All samples contained 2 mM DTT, 0.05 mg/ml BSA and 0.1 mM EDTA. The experimental protocol followed a standard method for measuring dissociation kinetics. BamHI (1.5 nM) was initially incubated with the 349 bp DNA fragment containing the specific BamHI recognition site (~3 nM) under conditions of virtually stoichiometric binding at 20°C for ~15 min. BamHI specific sequence oligonucleotide was then added to a final 200-fold excess concentration and the reaction mixture incubated for different times. The fraction of specifically bound BamHI was assayed using either the gel mobility shift technique or the self-cleavage assay. For the gel mobility shift assay, the stop reaction mixture was added and the samples were directly loaded on polyacrylamide gels. For the self-cleavage assay, the cleavage mixture also contains Mg$^{2+}$ in addition to osmolyte. Samples were incubated at 20°C for various times after adding the cleavage mixture. In kinetic experiments specific oligonucleotide was omitted from stop reaction and cleavage mixtures since 200-fold excess of the specific oligonucleotide was already present in the reaction mixture. DNA digestion products were purified using GenElute PCR Clean-up kit (Sigma Chemical Co. Inc.).

**Gel electrophoresis**

In the EcoRI gel mobility shift experiments (12–14) reaction mixtures were electrophoresed in a 10% polyacrylamide gel, TAE (22.5 mM Tris, 11.25 mM acetic acid and 0.5 mM EDTA (pH 8.3)) buffer. Samples were loaded on the gel at 130 V and the gel run for 40 min at this voltage. The voltage was then reduced to 50 V and the gel was run overnight at 20°C to separate free DNA fragments and EcoRI-bound complexes. EcoRI-specific DNA fragment complexes are remarkably stable in the polyacrylamide gels, no change in fractions of bound fragment was observed between 8 and 16 h run.

For BamHI gel mobility shift experiments, the electrophoresis buffer and gel contained 20 mM HEPES buffer (pH 6.7) and 2 mM CaCl$_2$. BamHI samples were loaded on the gel at 230 V, and the gel was run for 3–4 h at this voltage.

Ficoll was added to the DNA fragments from the self-cleavage assay to a final concentration of 3% and loaded on a 10% polyacrylamide gel, TAE (22.5 mM Tris, 11.25 mM acetic acid and 0.5 mM EDTA (pH 8.3)) buffer. Samples were run at 230 V for 3–4 h. Electrophoretic bands containing free DNA, and DNA–protein complexes were stained with the fluorescent dye SYBR Green I (Molecular Probes). The gels were imaged with a Luminescent Image Analyzer LAS-1000 plus (Fuji Film) that includes a 1.3 megapixel cooled CCD camera, epi-illumination at 470 nm (LED), and a dichroic optical filter suitable for SYBR Green I. The LAS-1000 plus was interfaced to a Pentium PC. Band intensities were quantified using Image Gauge (V.3.122) for Windows. The linearity of DNA fluorescent staining over the range of DNA concentrations studied was confirmed using pBR322 DNA fragments generated by MspI digestion.

**Equilibrium competition and kinetics data analysis**

As was developed previously (9), the ratio of specific (sp) and nonspecific (nonsp) association binding constants ($K_{sp}/K_{nonsp}$) can be determined from the loss of specifically bound complex as the concentration of a nonspecific oligonucleotide competitor DNA is increased. If $f_b$ and $f_{b0}$ are the fractions of protein-bound specific sequence fragment with and without added oligonucleotide competitor, then under conditions of virtually stoichiometric protein binding and for much weaker nonspecific than specific binding ($K_{nonsp} << K_{sp}$) the change in specific sequence binding is given by,

$$f_b = f_{b0} - \frac{K_{nonsp}}{K_{sp}} \frac{[DNA_{oligo}]_{total}}{1 - f_b} \frac{[DNA_{sp}]_{total}}{[DNA_{oligo}]_{total}}$$

Relative binding constants, $K_{sp}/K_{nonsp}$, were straightforwardly calculated from the linear dependence of $f_b$ on $[DNA_{oligo}]_{total}$, measured at constant specific sequence DNA and protein concentrations.

The dissociation kinetics of the specific protein–DNA fragment complex in the presence of a large excess of specific sequence containing oligonucleotide competitor can be well approximated by the irreversible first-order rate equation:

$$\frac{d[DNA_b]}{dt} = -k_{off}[DNA_b],$$

where $[DNA_b]$ corresponds to the concentration of complex and $k_{off}$ is the dissociation rate constant. The standard solution of this equation is,

$$\ln \left( \frac{f_b}{f_{b,0}} \right) = -k_{off}t,$$

where $f_b$ is the fraction of specific complex at time $t$ and $f_{b,0}$ is the fraction of complex at $t = 0$.

The dependence of $k_{off}$ on water activity can be calculated as change in the number of waters that exclude solute coupled to the dissociation of the specific complex (7,8,15),

$$\frac{d[ln(k_{off})]}{d[osmol]} = \frac{\Delta N_w}{55.6}$$

**RESULTS**

With the gel mobility shift assay, the equilibrium between free DNA and protein–DNA complex can be drastically disturbed between the time a sample is loaded and when it enters the gel. We have estimated the time it takes for DNA to enter the 10% polyacrylamide gel at 230 V by running the gel for fixed times before rinsing the solution remaining in the well. The experiment is illustrated in Figure 1. As can be seen on Figure 1B, only after ~5 min does DNA completely enter the gel. During this time the local pH and concentrations of salt, DNA, protein and complex can equilibrate with the running buffer and change substantially. Typically buffer conditions used in electrophoresis differ significantly from the solution conditions used for DNA–protein binding experiments. The ionic
strength of a TAE running buffer that is routinely used in gel electrophoresis (~10–20 mM) is much lower than ionic strengths typically used to prepare DNA–protein complexes. The pH of TAE buffer is 8.3, and buffers used to prepare DNA–protein complexes typically have lower pH (6.5–7.5). Importantly, the color of pH-sensitive indicator dyes added to the regular samples prior to loading them onto the gel changes within seconds of loading with an applied voltage (data not shown). If complex association and dissociation rates are faster than the minutes time scale for entering the gel then the initial equilibrium will be significantly perturbed.

The specific sequence binding constant of EcoRI is well known to be quite sensitive to salt concentration (16–18). In Figure 2A, reaction mixtures of EcoRI and a 360 bp DNA fragment containing the specific recognition sequence were equilibrated at different concentrations of NaCl (ranging from 90 to 240 mM NaCl) and loaded directly onto a 10% polyacrylamide gel at 130 V. Enough EcoRI was in the reaction mixture to bind a fraction f~0.65 of the DNA fragment at saturation. Only a weak salt dependence is observed; the fraction of EcoRI-bound DNA decreases from 0.63 at 90 mM NaCl to 0.52 at 240 mM NaCl. The dependence of fraction...
EcoRI-bound DNA fragment on NaCl concentration calculated from this gel is shown by triangles on Figure 2C. The apparent binding constants are very different from those reported using other techniques (16–18). Observed salt sensitivity translates into release of only ~2.5 ions in the binding reaction.

In Figure 2B, equilibrated samples were mixed with enough of the osmolyte betaine glycine to bring the final concentration to 2 osmolal and with a 200-fold excess of specific sequence oligonucleotide before loading the gel; samples were otherwise identical with those shown in Figure 2A. As shown previously (7), this concentration of betaine glycine decreases the dissociation rate constant of EcoRI–DNA complexes ~75-fold. It should also be noted that net neutral solutes as betaine glycine are significantly denser than water obviating the need for glycerol or ficoll in the binding reaction buffer. Nor will neutral osmolytes electrophorese away from the DNA samples in the well. The specific sequence oligonucleotide competitor ensures that free protein does not bind to free fragment DNA after ‘freezing’ the reaction with betaine glycine. Now a very strong salt dependence is observed. The fraction of EcoRI complex as a function of salt concentration using the stop reaction mixture is also shown in Figure 2C (circles). The salt sensitivity observed translates into ~9 ions released in the binding reaction, consistent with previously reported values (16–18). Without the stop reaction mixture, free protein that was present initially in the reaction mixture at high salt concentrations is binding to DNA fragment in the well before entering the gel, greatly altering the initial equilibrium as seen in Figure 2A.

Figure 3 shows that 2 osmolal concentration of betaine is high enough to stabilize the EcoRI-specific DNA fragment complex and preclude loss of initially bound protein when 200-fold excess of competitor specific site oligonucleotide is added to an equilibrium mixture of protein, DNA and complex. EcoRI and a specific site DNA fragment were incubated in 180 mM NaCl (pH 7.0) and at 20°C for a sufficient time to reach equilibrium. The triangle on the graph Figure 3B is the apparent fraction bound with no addition of the stop reaction mixture. Virtually all the free protein present initially in this sample binds DNA fragment before entering the gel due to equilibrium with the electrophoretic running buffer as in Figure 2A.

Stop reaction mixtures containing 200-fold excess of the specific sequence oligonucleotide and various concentrations of betaine glycine were added to the equilibrated samples before loading onto the gel (Figure 3A). Without betaine glycine but with specific oligonucleotide in the stop reaction mixture, virtually all the protein initially bound to the DNA fragment is lost to competing oligonucleotide, f_0 (~0. The dissociation rate of complex is too fast compared to the time needed to enter the gel. The observed fraction of complex increases with increasing betaine concentration and reaches a plateau value at ~2 osmolal betaine glycine. At this point the dissociation rate of the complex is slow enough that bound EcoRI is not lost by the complex before it enters the gel. In a control experiment, samples were prepared in 90 mM NaCl. This salt concentration is low enough that binding is essentially stoichiometric and only negligible differences in the fraction of the EcoRI-bound DNA fragment are observed with no specific oligonucleotide added, with the competitor added in the absence of betaine, and with the competitor and 2 osmolal betaine glycine added (data not shown). At 90 mM NaCl and pH 7.0 the half-life time of a specific EcoRI–DNA complex is ~40 min even without added osmolyte (7).

Self-cleavage assay

The ability to ‘freeze’ a reaction mixture has, of course, other applications. For site specific DNA binding proteins also possessing cleavage activity, such as restriction endonucleases, a self-cleavage assay allows measurement of their binding equilibrium and dissociation kinetics directly in solution without separating free protein, free DNA, and complex. Both EcoRI and BamH I restriction endonucleases, e.g. require Mg^{2+} as a co-factor to cleave DNA, but are capable of forming specific complexes with DNA in the absence of divalent ions. The same stop reaction mixture used with the gel mobility shift assay above can be used as the basis of a reliable self-cleavage assay. In this case, Mg^{2+} is added along with the competitor...
specific site oligonucleotide and osmolyte. The Mg\(^{2+}\) initiates the cleavage reaction. The osmolyte should be present in sufficient concentration to prevent dissociation of formed complexes before cleavage can occur. The competitor oligonucleotide should be present in sufficient concentration to trap all enzyme that is not only initially free but also released after subsequent cleavage of DNA fragments and oligonucleotides. Since osmolytes have also been reported to slow the rate of cleavage (19–22), trapping free enzyme will require less oligonucleotide in the presence of osmolyte than in its absence. A preferential DNA cleavage method has been used previously (17,23) to measure equilibrium binding and kinetics of EcoRI endonuclease, but without using neutral osmolytes to control dissociation rates.

**Self-cleavage assay applied to EcoRI-DNA binding**

Both specific site oligonucleotide concentration and neutral solute concentration (osmotic stress) are experimental parameters that can be varied to optimize the self-cleavage assay. One can verify that cleavage only occurs at those specific recognition sites that initially have bound enzyme by observing that the fraction of cleaved DNA does not change with time of incubation or that there is at least a well defined region of time with constant fraction cleaved. Figure 4A shows the time course for the fraction specific site fragment cleaved by the EcoRI after incubation at 20°C with 10 mM Mg\(^{2+}\) for several specific site oligonucleotide and betaine glycine concentrations. Enough EcoRI was added to the specific sequence 360 bp fragment to give an initial fraction bound of \(f_{b,0} \approx 0.5\). Samples were incubated for 15 min before the cleavage mixture containing MgCl\(_2\), betaine glycine and specific site oligonucleotide was added. The samples were then further incubated for various time intervals at 20°C. The cleavage reaction was then stopped by adding EDTA to a final concentration of 20 mM. The extent of fragment digestion was analyzed by gel electrophoresis (shown in Figure 4B). In the presence of 20-fold excess of specific oligonucleotide but without betaine present (Figure 4A, black circles), the DNA fragment is completely cleaved within 5 min incubation. With 0.44 osmolal betaine and 100-fold excess of the specific oligonucleotide (open triangles), the cleavage reaction is somewhat slowed, but still complete digestion of the specific site fragment occurs within \(\sim 30\) min. With 1.8 osmolal betaine and 100-fold excess of specific oligonucleotide (open squares), the fraction of specific DNA fragment cleaved by the EcoRI remains constant for at least 40 min. after adding MgCl\(_2\). In the presence of 4.4 osmolal betaine and 200-fold excess of specific oligonucleotide (black diamonds) fraction of specific DNA fragment cleaved by the enzyme remains unchanged for at least 1 h. Note that samples incubated with cleavage mix for shortest examined time, \(\sim 20\) s, showed fraction of cleaved DNA equal to fraction DNA initially bound.

Figure 5 shows a direct comparison of EcoRI binding analyzed by the gel mobility shift assay using the stop reaction and by the self-cleavage assay. Under the salt, temperature and pH conditions used, EcoRI binding is virtually stoichiometric. Both techniques give nearly same linear dependence of fraction bound DNA on the EcoRI concentration. Unless there is a coincidental and improbable cancellation of factors, both

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**Figure 4.** Both specific site oligonucleotide and neutral solute concentrations are parameters that can be varied to optimize the self-cleavage assay. EcoRI and a 360 bp DNA fragment containing its specific recognition sequence were initially incubated in the absence of Mg\(^{2+}\); a cleavage mixture containing an excess of 30 bp specific sequence oligonucleotide, MgCl\(_2\) and the osmolyte betaine glycine was then added to the pre-formed complex. Samples were incubated with cleavage mix for various time intervals at 20°C. The cleavage reaction was then stopped by adding EDTA to a final concentration of 20 mM. The extent of fragment digestion was analyzed by gel electrophoresis. (A) The time dependence of the fraction specific site DNA fragment cleaved by EcoRI after incubation at 20°C in 10 mM Mg\(^{2+}\) is shown for several specific site oligonucleotide and betaine glycine concentrations. Black circles—no osmolyte, [oligo]/[fragment] molar ratio = 20; open squares—0.44 osmolal betaine, [oligo]/[fragment] = 100; open squares—1.8 osmolal betaine, [oligo]/[fragment] = 100; black diamonds—4.4 osmolal betaine, [oligo]/[fragment] = 200. The solid line shows the fraction of DNA initially bound with the EcoRI measured by the gel mobility shift assay. (B) The fluorescent image of the gel corresponding to the cleavage experiment performed with 1.8 osmolal betaine and an oligonucleotide/fragment ratio of 100 is shown. The first lane shows the gel mobility shift assay of the initial EcoRI-DNA incubation with only stop reaction mixture added. In lanes 2–5, the initial reaction mixture of EcoRI and DNA was incubated after adding the cleavage mixture for the indicated times, the DNA fragments isolated, and then separated on the gel. EcoRI and DNA were initially incubated in 20 mM imidazole (pH 7.5) and 90 mM NaCl.
assays are quantitatively applicable to the measuring of the EcoRI-DNA binding.

**Self-cleavage assay applied to BamHI-DNA binding**

We have also examined a second restriction nuclease, BamHI. Essentially the same protocol is used as with EcoRI. In order to determine optimal conditions for specific site oligonucleotide competitor and osmolyte concentrations, BamHI was incubated for 15 min with a 349 bp fragment containing the specific BamHI site. MgCl₂, betaine glycine and BamHI specific site oligonucleotide were then added to the reaction mixture. The final concentration of Mg²⁺ was 10 mM. Samples were incubated for different time intervals at 20°C and the reaction then quenched by adding EDTA to a final concentration of 20 mM. Figure 6 shows that 2 osmolal betaine and 200-fold molar excess of the specific BamHI site oligonucleotide (over specific site 349 bp fragment) are sufficient to ensure that even after 2 h the fraction of cleaved fragment reflects the fraction of enzyme initially bound to the DNA. Other osmolytes such as triethylene glycol or methyl glucoside can be substituted for betaine glycine in the cleavage mixture with very similar results (data not shown). In all the BamHI self-cleavage assays described below, the solution conditions for the cleavage reaction were held constant: 10 mM MgCl₂, 200-fold excess of specific oligonucleotide, 2 osmolal betaine glycine and 20°C. The experiment illustrated in Figure 6 was performed at 100 mM KCl. In the presence of 60 mM KCl, the fraction DNA cleaved remains unchanged over at least 2.5 h as expected for conditions of even slower dissociation rates (data not shown).

**Self-cleavage and gel mobility shift assays applied to kinetics of BamHI specific complex dissociation**

Dissociation rates of the complex between BamHI and a 349 bp specific sequence fragment were measured using a standard approach. A 200-fold molar excess of specific sequence oligonucleotide was added to the pre-formed DNA–protein complex in 60 mM KCl and 0.6 osmolal betaine and the mixture incubated at 20°C for varying times. At this concentration of competitor oligonucleotide, re-association of free BamHI with the 349 bp DNA fragment after dissociation can be neglected. For each time point, 30 μl aliquots were taken from the sample and added either to 30 μl of cleavage reaction mixture for analysis by the self-cleavage assay or to 30 μl of a stop reaction mixture for the gel mobility shift assay. Since the reaction mixture already contains a high concentration of competitor oligonucleotide, neither the cleavage reaction mixture nor the stop reaction mixture contained additional specific sequence oligonucleotide. In order to stabilize the specific BamHI–DNA complex in the gel for the mobility shift assay, 2 mM CaCl₂ was added to the electrophoresis running buffer. Figure 7A shows gel images for the gel mobility shift and self-cleavage experiment. BamHI and DNA were initially incubated in 20 mM imidazole (pH 7.0) and 100 mM KCl.

Figure 5. A direct comparison of EcoRI-DNA binding analyzed by the gel mobility shift assay (black circles) using the stop reaction mixture (up to 200-fold of specific sequence oligonucleotide and 2 osmolal betaine glycine in the final sample) and by the self-cleavage assay (open triangles) using the cleavage reaction mixture (up to 10 mM MgCl₂, 200-fold excess of specific sequence oligonucleotide, and 2 osmolal betaine in the final sample). Both techniques give nearly same linear dependence of fraction bound DNA fragment on the EcoRI concentration. The initial EcoRI-DNA incubation salt conditions were 20 mM imidazole (pH 7.5) and 75 mM NaCl.

Figure 6. The experiment illustrated in Figure 4 was repeated for the restriction endonuclease BamHI to determine optimal conditions for the self-cleavage assay with this enzyme. The same protocol was used as outlined in the legend to Figure 4. Open circles—1.2 osmolal betaine, no oligonucleotide present; black circles—0.56 osmolal betaine, [oligo]/[fragment] = 100; open triangles—1.4 osmolal betaine, [oligo]/[fragment] = 50; black squares—1.2 osmolal betaine, [oligo]/[fragment] = 200; open diamonds—2 osmolal betaine, [oligo]/[fragment] = 200. In the presence of 2 osmolal betaine and 200-fold molar excess of the specific BamHI sequence oligonucleotide over specific site 349 bp fragment even after 2 h incubation with the cleavage mixture the fraction of cleaved fragment reflects the fraction of enzyme initially bound to the DNA (shown as dotted line) as determined by the gel mobility shift assay. BamHI and DNA were initially incubated in 20 mM imidazole (pH 7.0) and 100 mM KCl.
The self-cleavage assay was then applied to measure the dissociation rate of BamHI from its specific DNA sequence at different betaine concentrations. Figure 8 shows kinetic curves for the dissociation of BamHI as dependent on betaine glycine concentration measured in 60 mM KCl. Dissociation can be well described by a simple, single exponential process. Kinetic rate constants determined from the slopes vary from 0.107 min\(^{-1}\) at 0.2 osmolal betaine to 0.010 min\(^{-1}\) at 0.92 osmolal betaine. Figure 9 shows the dependence of the BamHI dissociation rate constant on betaine osmolal concentration. The half-life time of the specific complex increases \(\approx \)20-fold between 0 and 1 osmolal betaine in 20 mM imidazole (pH 7.0) and 60 mM KCl.

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The self-cleavage method was also used to measure the ratio of BamHI specific and nonspecific binding constants using a self-cleavage and gel mobility shift assays applied to measure specific versus nonspecific equilibrium competition binding of BamHI

The self-cleavage assay was then applied to measure the dissociation rate of BamHI from its specific DNA sequence at different betaine concentrations. Figure 8 shows kinetic curves for the dissociation of BamHI as dependent on betaine glycine concentration measured in 60 mM KCl. Dissociation can be well described by a simple, single exponential process. Kinetic rate constants determined from the slopes vary from 0.107 min\(^{-1}\) at 0.2 osmolal betaine to 0.010 min\(^{-1}\) at 0.92 osmolal betaine. Figure 9 shows the dependence of the BamHI dissociation rate constant on betaine osmolal concentration. The half-life time of the specific complex increases \(\approx \)20-fold between 0 and 1 osmolal betaine in 20 mM imidazole (pH 7.0) and 60 mM KCl.
DNA fragment is plotted against the parameter recognition sequence. The fraction of BamHI bound to specific sequence ing the nonspecific inverted sequence and a DNA fragment with the specific is extracted from the binding competition between an oligonucleotide contain-
sures as 4.9 (2/C6 competitor oligonucleotide were incubated in 20 mM imidazole (pH 7.0)
ombility shift assays, respectively. BamHI, DNA and nonspecific
nonspecific oligonucleotide in 60 mM KCl and 0.5 osmolal
direct competition assay. BamHI was added to the mixture of specific sequence fragment and different concentrations of nonspecific oligonucleotide in 60 mM KCl and 0.5 osmolal betaine, but without Mg^{2+}. BamHI binds specific DNA fragment stoichiometrically under these experimental condi-
tions. Samples were incubated for 150 min, long enough compared with the specific complex dissociation rate to ensure equilibrium. The stop reaction mixture or the cleavage reaction mixture was then added. The ratio of specific and nonspecific binding constants was extracted from the loss of specific sequence DNA fragment complex as the concentra-
tion of competitor nonspecific oligonucleotide increases according to Equation 1 in Materials and Methods. The results of the self-cleavage and gel mobility shift assays are shown in Figure 10. The ratio of specific and nonspecific association binding constants was measured as 4.9 (±0.7) × 10^3 and 4.5 (±0.2) × 10^3 for self-cleavage and gel mobility shift assays, respectively. BamHI, DNA and nonspecific competitor oligonucleotide were incubated in 20 mM imidazole (pH 7.0) and 60 mM KCl.

**DISCUSSION**

The use of separation techniques as filter binding, the gel mobility shift and other chromatography methods to probe equilibrium distributions requires that the equilibrium is not significantly disturbed during the separation process. We have focused on the gel mobility shift assay here, but the general principles are applicable to other techniques. For binding reactions maintaining the initial equilibrium distribution means that the dissociation and association rates of a complex are much slower than the separation time. Typically the separation itself precludes association. Once in the gel matrix, the dissociation of DNA–protein complexes is often quite slow due to caging or crowding effects (13, 24–26). The dissociation of complex within a gel can often be seen as a forward edge smearing of the complex band. By properly integrating over the smeared band the initial equilibrium distribution can be reconstructed (14). Until the various species actually enter the gel, however, the equilibrium distribution is particularly vulnerable due to changes in pH and salt concentrations caused by the electrophoresis of the buffer. We found (Figure 1) that at least 5 min are required at our experimental conditions for a DNA sample to fully enter the gel. We also found using pH-sensitive dyes that pH of the loaded sample starts to change immediately after voltage is applied. Samples mixed with a dense substance as glycerol or ficoll and loaded onto the well can be considered somewhat protected from simple diffusion (indeed without current pH of the sample can remain the same for minutes), but with applied voltage pH changes much faster due to the electrophoresis of buffer. This means necessarily that salt exchange is also quite fast.

The results shown in Figure 2 illustrate that initially free EcoRV will associate with DNA before entering the gel. This is in contrast to the more common emphasis given in the litera-
ture on the danger of disrupting DNA–protein complex and a subsequent underestimation of binding constants (4, 11). The binding of free protein before entering the gel is of particular concern if the free protein concentration is much larger than the concentration of DNA sites, as often is the case when the gel mobility shift assay is used to measure complete binding isotherms.

Figure 3 shows that simply adding competitor DNA to prevent binding of initially free protein to the target DNA, however, is not a complete solution. The dissociation rate of the complex is fast enough that virtually all the protein is lost from the initial target DNA complex to the competitor DNA before entering the gel. Only when enough neutral osmolyte is added to control the dissociation rate does the gel mobility shift assay reflect the initial equilibrium.

Interestingly, not only can solution techniques and the gel mobility shift assay sometimes give different results, but the gel mobility shift assay can also show quite different results depending on electrophoresis conditions. For example, Taylor et al. (1) and Vipond and Halford (2) concluded that the restriction nuclease EcoRV does not show any DNA sequence binding specificity in the absence of divalent ions using the gel mobility shift assay. Thielking et al. (27) also using gel mobility shift assay showed that a D90 mutant of EcoRV which is incapable of DNA cleavage binds a 20 bp oligonucleotide carrying specific site for the EcoRV ~100-fold stronger than nonspecific competitor in the absence of Mg^{2+}. Specific binding of the same mutant to a 377 bp fragment, however, could not be detected. In contrast, Engler et al. (4) reported about a 1000-fold preference for binding of wild-type EcoRV to the specific recognition sequence over nonspecific DNA sequences in the absence of divalent ions using both the gel mobility shift and filter binding assays. The running buffer of Engler et al. (4) had a pH 7 or lower (compared with pH 8–8.3 used by other authors). Engler et al. (4) concluded that at pH > 7 gel retardation significantly underestimates the binding association constant. Later Martin et al. (3) again using gel mobility shift assay reported that EcoRV binds to its specific sequence only 5-fold better than to nonspecific in the absence

![Figure 10](image-url)
of divalent ions, and ~10,000-fold better in the presence of Ca\(^{2+}\). Samples were prepared at pH 7.5, but gel was run at pH 7.0. Using fluorescence resonance energy transfer and fluorescence depolarization, Erskine and Halford (5) obtained identical equilibrium binding constants for EcoRV binding to specific and nonspecific sequences in the absence of divalent ions at pH 7.5. Reid et al. (6) measuring fluorescence anisotropy found that EcoRV preference to the specific sequence did not exceed ~6.5-fold in the absence of divalent ions and pH 7.5. The issue still remains unresolved.

We have taken advantage of our previous observation that neutral osmolytes can greatly slow the rate of DNA–protein complex dissociation to ensure that the complexes initially present do not dissociate before entering the gel. In order to prevent additional protein–DNA association before entering the gel, we add high concentrations of competitor oligonucleotide to trap free protein. For particularly labile complexes, osmolytes could even be added to the gel to stabilize complexes.

We have extended this general scheme to use the cleavage activity of restriction nucleases to measure the extent of binding with a self-cleavage assay. The binding reaction can be ‘frozen’ using the stop reaction mixture. Cleavage can be initiated by adding Mg\(^{2+}\). Products of the cleavage reaction are easy to purify, and DNA fragments can be run on a regular polyacrylamide or agarose gels. This self-cleavage technique allows measurement of binding equilibrium and kinetics of restriction endonucleases directly in solution avoiding intrinsic problems of the gel mobility shift and filter binding assays while providing the same sensitivity. The results shown in Figure 7 and 10 indicate that both the self-cleavage and gel mobility shift assays give the same results if used properly.

The osmolyte and competitor oligonucleotide concentrations of the cleavage mixture were optimized to ensure that fraction cleaved DNA reflects fraction DNA initially bound to protein by observing that the fraction cleaved is constant for at least 40 min. The original samples containing the DNA–protein complexes of interest before adding cleavage mix can of course be prepared at any salt or osmolyte concentration. The cleavage reaction mixture can be easily adjusted for each sample such that the final salt, osmolyte, oligonucleotide and pH conditions are identical.

The optimal osmolyte and oligonucleotide concentrations for the stop reaction or cleavage mixtures will, of course, vary depending on the application. Care should be taken with high osmolyte concentrations. Osmotic pressure will certainly alter the efficiency of filter binding and may promote promiscuous binding of protein and DNA. High osmotic pressures under conditions with significant concentrations of free protein may lead to association or aggregation of proteins. Control experiments are necessary to ensure there are no unintended consequences of high osmolyte concentrations.

The stability of restriction nuclease-DNA complexes can differ markedly. EcoRI–DNA specific complexes are remarkably stable in polyacrylamide or agarose gels. In contrast the specific complex of BamHI is significantly less stable in the absence of divalent ions. Engler et al. (11) have shown that the presence of Ca\(^{2+}\) does not support cleavage and increases the specific binding constant by ~250-fold. We find that the specific BamHI complex with 349 bp long DNA fragments in the absence of Ca\(^{2+}\) substantially dissociates in polyacrylamide gels particularly for BamHI specific sequences with non-optimal neighboring base pairs (11). The presence of 2 mM CaCl\(_2\) in the gel and in the running buffer is necessary to use the gel mobility shift assay confidently. The presence of divalent ions in the gel, however, decreases the overall quality of the gel image and quickly degrades platinum electrodes. Depletion of Ca\(^{2+}\) from the gel due to electrophoresis can result in destabilization of the complex during the course of an experiment.

The self-cleavage assay described here should be particularly useful for measuring binding reactions of those restriction enzymes that do not form very stable complexes with their specific sequences in the absence of divalent ions. We have demonstrated that the self-cleavage assay can be reliably used to measure both the dissociation kinetics and the equilibrium binding of BamHI. This technique is additionally attractive because of its simplicity and reproducibility. We have applied the self-cleavage assay to estimate the sensitivity of the dissociation rate of BamHI from its specific sequence to increasing concentrations of betaine glycine. The osmotic sensitivity of the dissociation rate to betaine glycine osmotic pressure seen in Figure 9 corresponds to an uptake of ~160 water molecules by the BamHI specific complex during dissociation. We have shown previously (7) that for a closely related restriction enzyme, EcoRI, the osmotic dependence of the specific dissociation rate is primarily due to the difference between specific and nonspecific binding of the protein. The uptake of 160 water molecules we measure for the BamHI specific dissociation is in a good agreement with the cavity size seen in the X-ray structure of the nonspecific BamHI–DNA complex (28) that is absent in the specific complex (29).

An osmotic sensitivity of the cleavage reaction has been already demonstrated for several restriction endonucleases including EcoRI, BamHI and PvuII (19–22) suggesting that the self-cleavage technique might be useful in general for measuring restriction endonucleases binding to DNA. In principle, the technique can be used for measuring binding or kinetics of any DNA binding protein that cleaves preferred sequences [such as, e.g. chimeric nucleases (30)] if their binding to DNA is sensitive to osmotic stress.

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