Macromolecular Hydration Changes Associated with BamHI Binding and Catalysis

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In this report, the effects of osmotic pressure on BamHI cognate binding and catalysis were investigated and compared with a previous study on EcoRI (Robinson, C. R. and Sligar, S. G. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 2186–2191). Our observation of the dependence of binding and catalytic parameters on osmotic pressure has allowed for the comparison of hydration changes associated with site-specific DNA recognition for both endonucleases. Over a large range of osmotic pressures ($\pi$), the dependence of BamHI on osmotic stress during cognate binding and catalysis was very different from that of the related endonuclease EcoRI. The binding of EcoRI to cognate DNA was dominated by a dehydration of the endonuclease-DNA complex, whereas binding by BamHI to its cognate sequence was accompanied by a solvent release corresponding to some 125 fewer waters. Catalytic analysis at elevated osmotic pressures indicated that both endonucleases had undergone a net hydration of the complex with BamHI displaying a much greater dependence on osmotic stress than EcoRI. Although the enzymes shared core structural motifs, comparisons of high resolution x-ray structures revealed many different secondary structural features of the complexed endonucleases. The large difference in hydration changes by both BamHI and EcoRI could be attributed to these dissimilar secondary structural features, as well as the functional differences of the two endonucleases during site-specific DNA recognition.

The BamHI endonuclease binds the DNA recognition sequence 5'-GGATCC-3' with remarkable specificity. The enzyme catalyzes double strand hydrolysis in the presence of divalent cations (Mg$^{2+}$, Mn$^{2+}$) after the first guanine base, leaving staggered 4-base pair (bp) overhangs (1–3). Previous studies that investigated the effects of increased osmotic stress on endonuclease specificity for both BamHI and EcoRI reveal that slight perturbations in water activity could cause the enzymes to lose specificity for their cognate DNA recognition sequences (4–6). Hence, in the presence of cosolvents, BamHI and EcoRI have a propensity to cleave at non-cognate or “star” sites which are characterized by base pair (bp)$^1$ changes within the hexameric cognate recognition sequence. In these studies which employed added solutes to modulate the various colligative properties of the reaction solution, increase in the observed star activity was only correlated with osmotic pressure ($\pi$) and was not correlated with other solution properties, such as dielectric constant, viscosity, and mole fraction of water (4, 6).

A key experiment in determining whether these effects were linked to changes in hydration was through the reversion of the observed star activity with the application of hydrostatic pressure (5, 6). Hydrostatic pressure has been shown to preferentially hydrate macromolecules and biomolecular complexes (7, 8). The stringent molecular recognition of the endonucleases was restored by the application of hydrostatic pressure under star conditions, presumably through the replacement of waters released at elevated osmotic stress levels. This led to the conclusion that the observed star activity was because of increased osmotic stress from the added solutes, apparently through the removal of key water molecules or the hydration-linked conformational changes that are central to site-specific recognition.

Osmotic pressure has been a widely used technique to measure the changes in macromolecular hydration and to investigate the relationship between hydration and function (9, 10). This methodology relies on the exclusion of solutes from water associated with proteins or macromolecular complexes and a difference in the number of solute-excluding waters between two conformations of the protein or the complex (11). The concepts of crowding and preferential hydration, however, were developed to explain the exclusion from exposed surfaces. Crowding (12) suggests that exclusion is dependent on osmolyte size and geometry, whereas preferential hydration (13) adds that exclusion should also depend on the chemical nature of the osmolyte. The pictures of crowding and preferential hydration focus on the properties of solutes that are excluded, whereas osmotic stress emphasizes the role of water that is necessarily included if solutes are excluded (14, 15).

The difference in solute concentrations between the bathing solution and water-filled cavities is thermodynamically equivalent to an osmotic pressure that acts to favor the state with the smallest volume, thereby releasing water. The inference of a change in bound water through the dependence of binding-free energy on the water chemical potential is analogous to the measurement of ion release through the dependence of binding constants on salt activity, protonation through pH sensitivity, or entropic factors through temperature dependence of rate processes (15).

The dependence of binding and catalytic parameters on osmotic pressure allows the determination of solvation change associated with each biomolecular event. Therefore, the correlation between osmotic pressure and a given recognition event can provide insight into the role of water during that particular process (16). Several biological reactions, such as the opening/closing of membrane-bound channels (17), electron transfer of
cytochrome oxidase (18), glucose binding by hexokinase (19), oxygenation of hemoglobin (20), and DNA binding by several protein systems (9, 16), have also been the subject of studies in which osmotic stress was applied to measure macromolecular hydration changes. In these nucleic acid-protein interactions, the measured volume changes offer insight into the importance of hydration of the protein-DNA complex and thermodynamic consequences of solvent mobilization during specific endonuclease cleavage.

In this study, we focused on the observed differences in the solvation of specific endonuclease-DNA complexes during binding and cleavage of DNA by two restriction enzymes, BamHI and EcoRI. It was interesting to compare the solvation changes of BamHI and EcoRI because of their structural and functional similarities. The core structure of both endonucleases consists of five β-sheets surrounded by two α-helices that share a striking resemblance to one another (21). Functionally, the DNA cleavage products of both enzymes are staggered 4-bp overhangs, in which catalysis is thought to occur through sequential action by the monomeric subunits. Because of the conserved structure and function of both endonucleases, it might be anticipated that hydration changes during specific recognition events would be similar. However, when we measured the effects of osmotic pressure on specific DNA binding and catalysis by BamHI with ethylene glycol and betaine, significant qualitative and quantitative differences were observed. The origin of the differences in solvation changes observed during site-specific binding and catalysis by BamHI and EcoRI is discussed below.

**EXPERIMENTAL PROCEDURES**

**Materials**

T<sub>4</sub> polynucleotide kinase was purchased from Life Technologies, Inc., BamHI was purchased from New England Biolabs and used without further purification. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and purified separately by polyacrylamide gel electrophoresis. [γ-<sup>32</sup>P]-ATP (5000 Ci/mmole) was purchased from Amersham Pharmacia Biotech. The reaction buffer for binding assays contained 10 mM Tris-HCl, 50 mM NaCl, 100 μg/ml bovine serum albumin, 0.1 mM EDTA, 1 mM dithiothreitol, pH 7.4. All reagents were of the highest available purity.

**Methods**

*Substrate DNA and Enzyme—*Two complementary strands of DNA containing the BamHI cognate recognition sequence (5′-CTCGTATATGGAGATCCGCAGTAAGCT-3′) were 5′ end-labeled with [γ-<sup>32</sup>P]-ATP by T<sub>4</sub> polynucleotide kinase as described previously (22). The labeled DNA strands were extracted with phenol-chloroform-isomyl alcohol (24: 25:1) and were passed through a Bio-Rad BioSpin column to separate the labeled strands from the unincorporated nucleotides. The concentrations of the DNA oligonucleotide were measured spectrophotometrically. The complementary oligonucleotides were annealed by mixing equimolar concentrations of the DNA strands, by heating the mixture to 90°C, and by allowing it to cool slowly to room temperature. BamHI cleavage concentration was determined with the Bio-Rad Protein Assay kit by using bovine serum albumin as standard.

*DNA Binding Assays—*Equilibrium DNA binding assays were performed in the reaction buffer at 25°C by incubation of the <sup>32</sup>P-labeled DNA (1 pM) with varied concentrations of BamHI for at least 30 min. ethylene glycol or betaine was added to each buffer in a concentration range from 0 to 4M to achieve the desired osmolalities. Osmotic pressures (σ) were determined as described previously (23). The samples were loaded onto 15% polyacrylamide gels (37.5:1) in 0.5× TBE buffer and run at 12 V/cm. Gels were fixed, dried, and exposed by using a PhosphorImager screen (Molecular Dynamics). Band intensities of the DNA substrate and cleavage products were also quantified by using ImageQuant software (Molecular Dynamics) with the volume measurement utility. Reaction initial velocities (V<sub>0</sub>) were determined from the initial linear portion of the reaction curve according to Equation 2.

\[
V_0 = k_{cat}[E-DNA]_0 (Eq. 2)
\]

where [E-DNA]<sub>0</sub> is the fraction of the DNA product, [DNA]<sub>0</sub> is the initial DNA-substrate concentration, and f is time. Values for k<sub>cat</sub> were calculated by using Equation 3.

\[
V_0 = k_{cat}[E-DNA] (Eq. 3)
\]

where [E-DNA] equals the concentration of the enzyme-DNA complex. Each cleavage assay was performed at least four times for each osmotic pressure tested.

**Volume Determination—**Equilibrium volume changes (ΔV) and activation volumes (ΔV<sub>A</sub>) were calculated using the slopes of plots ln(K<sub>d</sub>/[E]) versus [E]<sub>0</sub> (Fig. 1) versus osmotic pressure following the relationship in Equations 4 and 5.

\[
\ln K_d/\Delta V = \Delta V/RT (Eq. 4)
\]

or

\[
\ln k_{cat}/\Delta V = \Delta V/RT (Eq. 5)
\]

where V is osmotic pressure, R is gas constant, and T is temperature.

**RESULTS**

Gel mobility shift assays were performed to measure the affinity of the BamHI endonuclease for the cognate recognition sequence under a large range of osmotic pressures (Table I). The binding isotherms were fit to Equation 1, and the equilibrium dissociation constant at several osmotic pressures was determined (Fig. 1). The dissociation constant determined at ambient pressure agrees with earlier results (24). Unlike the
EcoRI endonuclease, BamHI does not display a large increase in affinity towards the cognate recognition sequence as the osmotic stress is increased. Instead, the observed dissociation constant for BamHI with its cognate recognition sequence shows only a small increase in affinity with added osmotic stress. Whereas the dissociation constant for EcoRI binding to its cognate recognition sequence was lowered 200-fold at 50 atm osmotic pressure, the $K_{d}$ for BamHI binding to its cognate recognition sequence was lowered only 5-fold at 100 atm for both osmolytes tested (Table I).

The volume changes associated with endonuclease binding to DNA were calculated by plotting the ratio of the $K_{d}$ at ambient and osmotic pressures as a function of osmotic pressure. The slope of the line, according to Equations 4 and 5, is the volume change ($\Delta V$) associated with the cognate binding by BamHI (Fig. 2). A value less than zero for this slope indicated solvent release or net dehydration of the recognition complex. The measured volume change for BamHI cognate binding using ethylene glycol ($-370 \pm 20$ ml/mol) and betaine ($-450 \pm 50$ ml/mol) were considerably less than the volume change for EcoRI (Table II).

These results indicated a release of water molecules on specific DNA binding of both endonucleases, with EcoRI releasing a significant amount more than BamHI.

The observed reaction rates for the cleavage at the cognate recognition sequence by BamHI were found to be severely decreased at elevated osmotic pressures (Fig. 3). Over the range of 0–50 atm, the measured catalytic rate ($k_{cat}$) of BamHI was decreased by more than 25-fold (Fig. 4). In contrast, the reaction rate for EcoRI cleavage at the cognate site was decreased only by about 5-fold at $\pi = 100$ atm (Table III) (23). To ensure that the cleavage reactions were performed under actual maximal velocity conditions, the substrate concentration was doubled at each osmotic pressure and was found to have no effect on the observed reaction rate. The dependence of catalytic rate constants on osmotic pressure by using ethylene glycol and betaine as the cosolvents was similar.

The calculation of the activation volume change ($\Delta V^*$) associated with cognate catalysis, following Equations 4 and 5, revealed another large difference in the solvation change between BamHI and EcoRI. As seen in Fig. 5 the slope of the line for the activation volume determination was much greater for BamHI than for EcoRI cognate catalysis. The negative slope of the line for the $\Delta V^*$ determination indicated a net solvation of both complexes during catalysis. Whereas the activation volume for EcoRI at the cognate sequence was $-490 \pm 20$ ml/mol, the measured activation volume using ethylene glycol was $-1540 \pm 70$ ml/mol and using betaine was $-1640 \pm 70$ ml/mol for the BamHI cognate catalysis (see Table II).

**DISCUSSION**

We have shown that the binding of cognate DNA by BamHI is accompanied by little dehydration of the macromolecular complex, whereas the binding of EcoRI is dominated by dehydration that is almost an order of magnitude larger. Also, the similarity of the measured BamHI hydration changes using ethylene glycol and betaine suggests that the specific endonuclease-DNA complex does not expose any new surface area in response to the presence of solutes with different physical and chemical properties. This large observed difference in hydration between the two specific complexes is surprising considering the core structural similarities of both endonucleases.

The secondary structures of BamHI and EcoRI are characterized as $\alpha/\beta$ structures, built around a central mixed $\beta$-sheet with surrounding $\alpha$-helices (21). The common core motif of both endonucleases, when superimposed on one another, has a root mean square deviation of about 2 Å. The endonucleases also share similar values for buried surface area when complexed to cognate DNA. Previous studies suggest that the water release during EcoRI recognition is because of dehydration of the en-

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**Fig. 1. Osmotic pressure effect on the binding of BamHI to its cognate recognition sequence.** Representative binding isotherms for BamHI cognate gel shift analysis at ambient (▲) and 100 atm osmotic pressure (■), by using ethylene glycol as the added solute.

**Fig. 2. Volume change associated with BamHI and EcoRI binding of the cognate DNA recognition sequence.** The symbols represent the natural log of $K_{d}$ at ambient (0) and osmotic pressure ($\pi$). The slope of the line for recognition at the cognate site for BamHI is 0.0151 ($\pm 0.001$) atm$^{-1}$ for ethylene glycol (●) and 0.0185 ($\pm 0.002$) atm$^{-1}$ for betaine (▲). For comparison, the data for EcoRI (■) are shown where the slope is 0.108 ($\pm 0.003$) atm$^{-1}$.

**Fig. 3. Kinetic analysis of BamHI.** A representative polyacrylamide gel of the reaction progress of BamHI catalysis. Cleavage at the cognate recognition sequence of a DNA 26-mer at both A, ambient pressure, and B, 50 atm osmotic pressure, by using ethylene glycol as the added solute.

**Table II**

| Endonuclease | Dissociation ($\Delta V$) | Turnover ($\Delta V^*$) |
|--------------|--------------------------|------------------------|
| BamHI-EGOH   | $-370 \pm 20$ (20)       | $-1540 \pm 70$ (70)    |
| BamHI-Betaine| $-450 \pm 50$ (50)       | $-1640 \pm 70$ (70)    |
| EcoRI*       | $-2640 \pm 70$ (70)      | $-490 \pm 20$ (20)     |

* Taken from Ref. 23.

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Hydration Changes Associated with BamHI Cognate Recognition

![Graph](image)

**FIG. 4. Osmotic pressure effect on catalytic rate constant ($k_{cat}$).** Initial velocity kinetic profiles for BamHI cleavage at the cognate DNA sequence. The range of pressures measured, 0 (△), 10 (●), 20 (■), 30 (▼), 40 (○), and 50 (■) atm, are distinguished by different symbols representing experimental data points. The osmolyte used represented by this illustration was ethylene glycol.

**TABLE III**

| Osmotic pressure (atm) | Ethylene glycol | Betaine | EcoRI‡ |
|------------------------|-----------------|---------|-------|
| 0                      | 2.6 (±0.4)      | 2.6 (±0.4) | 5.0 (±2.0) |
| 10                     | 1.4 (±0.2)      | 1.3 (±0.1) |     |
| 20                     | 0.6 (±0.1)      | 0.3 (±0.05) | 3.3 (±1.1) |
| 30                     | 0.4 (±0.07)     | 0.3 (±0.05) |     |
| 40                     | 0.3 (±0.05)     | 0.2 (±0.05) | 2.2 (±1.0) |
| 50                     | 0.1 (±0.02)     | 0.1 (±0.01) |     |
| 60                     | 0.1 (±0.02)     | 0.1 (±0.01) |     |
| 80                     | 0.9 (±0.3)      | 0.9 (±0.3) |     |
| 100                    | 0.9 (±0.3)      | 0.9 (±0.2) |     |

‡) Taken from Ref. 23.

![Graph](image)

**FIG. 5. Activation volume change associated with BamHI and EcoRI catalysis at the cognate recognition sequence.** The symbols represent the natural log of $k_{cat}$ at ambient (0) and under osmotic pressure (atm). The slope of the line for cleavage at the cognate site for BamHI is −0.063 (±0.003) atm⁻¹ for ethylene glycol (○) and −0.067 (±0.003) atm⁻¹ for betaine (△). For comparison, the data for EcoRI (■) are shown where the slope is −0.020 (±0.001) atm⁻¹.

donuclease-DNA interface in which a hydration layer is removed during specific binding (15, 25).

Inspection of the high resolution x-ray crystal structures of the complexes of endonucleases with cognate DNA revealed key differences in the secondary structural elements of both endonucleases outside the common core motif. Although BamHI appears to be a more compact enzyme, EcoRI possesses inner and outer “arms” located near the recognition core (26, 27), which give the appearance of EcoRI surrounding the DNA recognition sequence when complexed with cognate DNA. An important difference between EcoRI and BamHI recognition is that the target DNA is “kinked” roughly 50° when bound by EcoRI, which is necessary for inclusion of the base recognition elements into the major groove (27, 28). The energy of the DNA kink has been estimated at a free energy cost of about 31 kcal/mol (29).

Investigations of the thermodynamic contributions of specific DNA binding by EcoRI, such as the release of bound water from nonpolar surfaces (hydrophobic effect), phosphate contacts, DNA distortion, and protein conformation, reveal a large need for favorable free energy to compensate for the significant DNA and protein structural changes during recognition (30). In addition, the formation of the specific EcoRI-DNA complex is characterized by a large negative heat capacity ($\Delta C_p$) (31). Record and co-workers (31) make the assumption that the large negative heat capacity is solely derived from the release of ordered waters from nonpolar surfaces (−120 kcal/mol), whereas Jen-Jacobsen (30) estimates a contribution of −60 kcal/mol for the hydrophobic effect. Although there are some uncertainties to the exact value, both of these investigations suggest that water release plays a large part in the specific binding of EcoRI. However, the binding of the cognate recognition sequence by BamHI is not accompanied by a bend in the DNA as revealed through high resolution structural analysis or through a large release of waters. We propose that the large differences in hydration during EcoRI and BamHI cognate binding are manifested in the free energy required for the conformational rearrangements associated with EcoRI-DNA specific binding.

The observed activation volume during cognate catalysis by both BamHI and EcoRI is also quite different. Both endonucleases share conserved active sites, and it has also been suggested that this conservation of active site residue coordination can be attributed to the staggered 4-bp overhang products of both enzymes (21). Although both have undergone a net hydration during cleavage, the measured activation volume of BamHI was observed to be 3 times that of EcoRI. The net hydration accompanying formation of the endonuclease-DNA complexes indicates that the rate-limiting step for both endonucleases is either product release or transition to a more “loosely” bound state.

Recently, Viadiu and Aggarwal (32) have focused their efforts on forming crystals of BamHI trapped in a transitional state to dissect the mechanism and role of active site residues during cognate site catalysis. These investigators soaked the BamHI-DNA cocrysals in Mn²⁺ with the intent of displacing the Ca²⁺, that was present in the crystal growth solution. The cocrysals appeared to crack at the beginning of the soak but eventually “healed” to form an intact crystal. Surprisingly, Mn²⁺ only displaced the Ca²⁺ ion in one subunit of the dimer, and phosphodiester bond hydrolysis was observed only in the subunit that bound Mn²⁺. Also, the Mn²⁺-bound subunit possessed an unrueled helix (α7), which uncoils upon binding and is situated in the DNA minor groove (32, 33). Viadiu and Aggarwal (32) thus suggest that the subunit that binds the minor groove begins DNA strand hydrolysis first, and then the other subunit undergoes this observed structural change in a sequential manner to complete double strand cleavage.

The trend associated with the solvation of the BamHI catalytic complex, which is observed, is consistent with the rate-limiting step of product release or transition from a tightly bound mode to a looser complex. The applied osmotic stress may also disrupt the transitional state of the sequential unfolding of helix-α7 in each BamHI monomer, which would explain the drastic differences in activation volume ($\Delta V$) be-
tween EcoRI and BamHI. However, this is not the case as our single turnover catalytic measurements at elevated osmotic stress levels reveal no difference in the first and second strand hydrolysis rates (data not shown). The increased sensitivity to osmotic stress by BamHI versus EcoRI can be explained through the mechanisms to which both enzymes complete double-stranded DNA cleavage and release product. Single turnover analysis of EcoRI shows that the rate for first strand hydrolysis is slower than that of second strand hydrolysis, which, on one hand, indicates that occasionally the nicked intermediate is released by the endonuclease and is rebound to a complete double strand cleavage (34). On the other hand, first strand is slower than second strand hydrolysis in BamHI, signifying that the endonuclease is more likely to complete double-stranded cleavage when a recognition complex is formed (35). If the rate-limiting step for both endonucleases is product release, the difference in turnover rate at elevated osmotic stress levels can be attributed to the preference of BamHI to remain bound to both the nicked and doubly cleaved product.

In summary, whereas the restriction endonucleases BamHI and EcoRI both possess similar core structural features, they do not share similar hydration properties during site-specific processes. We explain these findings through observed functional differences between the two enzymes upon specific recognition. The comparison of hydration properties of biological objects by using both experimental and structural data may be complicated by the use of cosolvents (i.e. polyethylene glycol, glycerol) used in the growth of crystals for x-ray structure determination. It is, therefore, important for continued solution structural investigations, which focus on the solvation of endonucleases and target DNA at elevated osmotic stress levels, to document key steps in the stepwise cleavage of DNA by the restriction endonucleases. Studies that incorporate the osmotic stress methodology help to gain insight into the role of water during site-specific recognition, and these studies have become an important tool for understanding hydration changes during biomolecular events.

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