

## Dissecting the Molecular Origins of Specific Protein-Nucleic Acid Recognition: Hydrostatic Pressure and Molecular Dynamics

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**ABSTRACT** The fundamental processes by which proteins recognize and bind to nucleic acids are critical to understanding cellular function. To explore the factors involved in protein-DNA recognition, we used hydrostatic pressure to perturb the binding of the *Bam*HI endonuclease to cognate DNA, both in experiment and in molecular dynamic simulations. A new technique of high-pressure gel mobility shift analysis was used to test the effects of elevated hydrostatic pressure on the binding of *Bam*HI to its cognate recognition sequence. Upon application of a pressure of 500 bar, the equilibrium dissociation constant of *Bam*HI binding to the cognate site was found to increase nearly 10-fold. A challenge has been to link this type of pure thermodynamic measurement to functional events occurring at the molecular level. Thus, we used molecular dynamic simulations at both ambient and elevated pressures to reveal details of the direct and water-mediated interactions between *Bam*HI and cognate DNA, which allow explanation of the effects of pressure on site-specific protein-DNA binding and complex stability.

### INTRODUCTION

The restriction endonuclease *Bam*HI recognizes the sequence 5'-G'GATCC-3' (cognate recognition sequence) with remarkable specificity and catalyzes double-strand DNA hydrolysis after the first 5'-G in the presence of divalent cations ( $Mg^{2+}$ ) (Wilson and Young, 1975; Aggarwal, 1995). Several x-ray crystallographic studies have been performed on the *Bam*HI-DNA complex to identify the key interactions involved in site-specific recognition. Comparison of the x-ray structures of the homodimeric *Bam*HI bound to a twelve-basepair DNA oligonucleotide (Newman et al., 1995) and that of free protein (Strzelecka et al., 1994; Newman et al., 1994) reveals protein structural changes that are associated with specific recognition. The most prominent structural change involves the unfolding of the C-terminal  $\alpha^7$  helix (residues 194 to 213) of both monomers. These residues comprise a region referred to as the "recognition arm." The "arm" from one monomer folds back toward the protein core structure and makes contacts with the phosphate backbone, whereas the "arm" from the other monomer inserts into the DNA minor groove. In the latter case, this part of the structure makes direct contacts with the DNA through residues Gly<sup>197</sup>, Met<sup>198</sup>, and a water-mediated contact through Asp<sup>196</sup>. This arm is an important recognition element, as proven by a study of the cognate *Bam*HI-DNA crystal soaked with  $Mn^{2+}$  to initiate DNA cleavage (Viadu and Aggarwal, 1998). Surprisingly, cleav-

age was observed only in the DNA strand bound by the monomer with the arm inserted in the DNA minor groove.

Although many factors are involved in site-specific recognition, it is well documented that water molecules play a key role in both the stability and specificity of protein-nucleic acid interactions (Schwabe, 1997). Water molecules are often resolved in protein-DNA crystal structures and identified as mediators of specific interactions (Otwinowski et al., 1988; Lawson and Carey, 1993). Structural studies alone, however, do not describe the additional events that take place during site-specific protein-DNA binding. The role of hydration on protein-DNA binding equilibria is also important because of the paramount thermodynamic contributions that water molecules contribute to complex stability (Schwabe, 1997). Previous thermodynamic investigations have shown that sequence-specific DNA binding is often accompanied by a large and negative change in heat capacity ( $\Delta C_p$ ) (Ha et al., 1989). This is generally taken as an indication of the removal of solvent-accessible surface from bulk water upon complexation and the formation of a highly complementary interface. The combination of structural studies with detailed thermodynamic analyses is therefore important in understanding the physical basis for sequence specificity and stability in protein-nucleic acid systems (Marky and Breslauer, 1987).

An important means to investigate the role of water molecules during protein-DNA binding is through the use of hydrostatic pressure. Hydrostatic pressure acts to hydrate protein-nucleic acid complexes, thus shifting the equilibria toward the state with least occupied volume (Parsegian et al., 1995; Robinson and Sligar, 1995a). Protein-DNA binding equilibria respond to pressure in a way defined by the size and sign of their volume change. The dependence of binding equilibrium on pressure can be used to extract volumetric information of the biomolecular complex ( $\Delta V$ )

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and lend insight on the process of specific *Bam*HI-DNA binding.

Methods used for the quantification of protein-DNA binding equilibria include fluorescence energy transfer and anisotropy (Heyduk and Lee, 1990; Royer et al., 1990), fluorescence intensity (Draper and Gold, 1980), calorimetry (Ladbury et al., 1994), and gel mobility shift assays (Fried, 1989; Carey, 1991; Fried and Bromberg, 1997). Gel mobility shift analysis offers the distinct advantage of direct visualization of pressure effects on the protein-nucleic acid system. The mobility shift assay consists of adding a DNA binding protein to a solution containing a DNA oligomer, containing a recognition sequence, which can be specifically bound by the protein. After equilibration at elevated pressure, protein-DNA complexes are separated from the free DNA probe by electrophoresis in the polyacrylamide gel. The resolved species are visualized and the stoichiometries of each species are determined to measure binding affinities.

An electrophoresis vessel, capable of maintaining hydrostatic pressures up to 2 kbar, was constructed for the purpose of performing high-pressure gel mobility shift assays to monitor the effects of pressure on the specific *Bam*HI-DNA complex. The apparatus is similar to the flatbed apparatus of Paladini (Paladini et al., 1987), which used high-pressure electrophoresis to affect the pressure dissociation of multimeric proteins (Paladini et al., 1994).

The difficulty with any type of thermodynamic measurement is the ability to assess the structural origin of the observed pressure effects. Molecular dynamics (MD) simulations are an important and widely used theoretical tool for modeling detailed micro- and macroscopic behavior of protein-nucleic acid systems (Bishop and Schulten, 1996; Kosztin et al., 1997; Sen and Nilsson, 1999). Simulations of these complexes provide insight into their structural, dynamical, and thermodynamic properties. Additionally, this method gives atomic-level insight into the short-time scale (pico seconds to nanoseconds) dynamics typically masked by averaging in x-ray crystallographic experiments. Therefore, to localize the origin of the thermodynamic effects observed in the high-pressure binding assays, extended time MD simulations were performed on the cognate *Bam*HI-DNA complex at both normal and elevated pressures.

Starting from the crystal structure of the *Bam*HI-DNA complex (Protein Data Bank entry 1bhm) (Newman et al., 1995), a system containing the *Bam*HI protein, solvent, and cognate DNA was constructed and simulated at both ambient and elevated pressure. Analysis of these simulations was focused on structural changes and comparison to direct, as well as water-mediated contacts between protein and DNA. The simulations were not intended to describe the effects of pressure on the mobilities of both the free DNA and *Bam*HI-DNA complex, as they are not affected by the modest pressures used in the high-pressure binding assay. The simulations revealed the pressure-induced structural perturba-

tions and the specific interactions responsible for *Bam*HI-DNA stability at elevated pressure.

## MATERIALS AND METHODS

### High-pressure gel mobility shift assays

DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and purified separately by polyacrylamide gel electrophoresis. Two complementary strands of DNA containing the *Bam*HI cognate recognition sequence (5'-CTCGTATAATGGATCCGAGTA-AGCT-3') were 5' end labeled with [ $\gamma$ - $^{32}$ P]ATP by  $T_4$  polynucleotide kinase. The labeled DNA strands were purified and annealed according to a previous study (Robinson and Sligar, 1998). *Bam*HI was purchased from New England Biolabs (Beverly, MA) and was used without further purification. Binding assays were performed in the reaction buffer at 25°C by incubation of the  $^{32}$ P-labeled DNA (1 pM) with varied concentrations of *Bam*HI. Reaction buffer for binding assays was the same as previously described (Lynch and Sligar, 2000). The samples were loaded onto 15% polyacrylamide gels (37.5:1) in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA) buffer, the electrophoresis vessel was pressurized, and the gels were run at 9 V/cm. Gels were fixed, dried, and exposed using a Molecular Dynamics phosphorimaging screen.

### Data analysis

Band intensities of the complexed and uncomplexed DNA were quantified using ImageQuant software with the volume measurement utility. The equilibrium constant ( $K_d$ ) was determined as described previously (Robinson and Sligar, 1998), using the relationship:

$$\Theta^{-1} = 1 + (K_d/[E_t]) \quad (1)$$

where  $\Theta$  is the fraction of bound DNA,  $E_t$  equals total enzyme concentration, and  $K_d$  is the equilibrium dissociation constant. Each binding assay was performed at least four times for each pressure tested. The volume change of dissociation was determined using the relationship:

$$\delta K_d/\delta P = -\Delta V/RT \quad (2)$$

where  $P$  equals pressure,  $V$  is the volume change,  $R$  is the universal gas constant, and  $T$  is the temperature.

### MD simulations

The protein-DNA system was built using the crystal structure of the cognate *Bam*HI-DNA complex (entry 1bhm in the Protein Data Bank) (Newman et al., 1995). The DNA contained 11 basepairs with the overhanging 5' thymine base removed. Missing residue atoms in the protein were modeled using X-PLOR (Brünger, 1992). The protein-DNA system was energy minimized using the Powell algorithm to remove unfavorable contacts and reduce the strain in the system. A pre-equilibrated cube of water molecules ( $88 \times 88 \times 88$  Å dimensions) was superimposed on the protein-DNA system for hydration and water molecules closer than 2.4 Å to the protein-DNA system were removed. Sodium ions were added by replacing water molecules to bring the resulting protein-DNA-solvent system to charge neutrality. This system contained ~65 300 atoms and, after equilibration at 297 K, was simulated under ambient and elevated pressure for 1 ns. In the simulation at elevated pressure a gradient of 50 bar/100 ps was used until a pressure of 400 bar was attained. The MD simulations were carried out using the program NAMD2 (Nelson et al., 1996), with v.26 of the CHARMM force field (MacKerrel et al., 1992). All hydrogen bonds were constrained during the simulations using SHAKE and an integration time-step of 2 fs was used. The system was simulated in

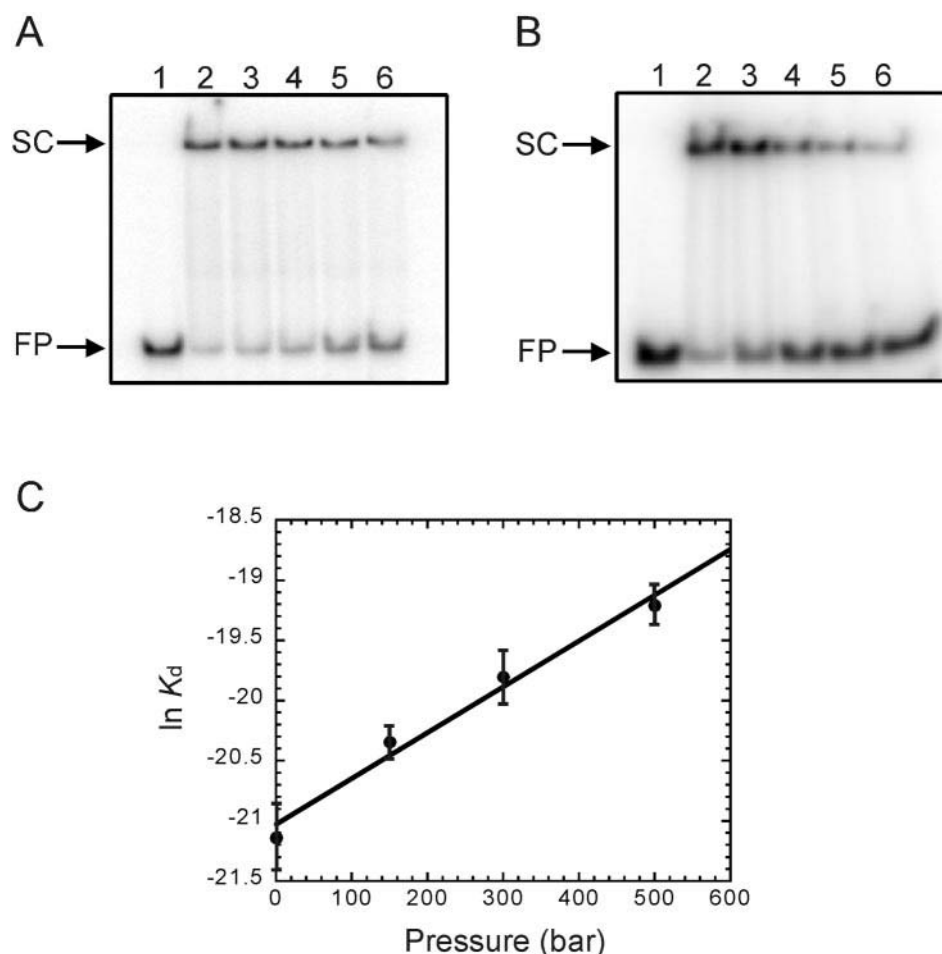


FIGURE 1 High-pressure gel mobility shift analysis. (A) *Bam*HI binding a DNA 26-mer containing the cognate recognition sequence at ambient pressure. Lane 1 represents DNA alone; *Bam*HI is added to the samples represented in lanes 2–6 in decreasing concentrations starting from lane 2. (B) *Bam*HI binding the DNA 26-mer at an elevated hydrostatic pressure of 300 bar. The *Bam*HI titration is the same as represented in A. SC, specific *Bam*HI-DNA complex. FP, free DNA probe. (C) Shown is the dependence of the *Bam*HI-DNA equilibrium dissociation constant ( $K_d$ ) as hydrostatic pressure is increased.

NpT ensemble mode with periodic boundary conditions and full electrostatics computed using the particle mesh Ewald method (Luty et al., 1994). Constant pressure was maintained using the Langevin piston method (Feller et al., 1995) with a piston period of 200 fs, a damping time constant of 100 fs, and a piston temperature of 297 K. Langevin dynamics was used throughout both 1-ns simulation runs.

## RESULTS

### High-pressure gel mobility shift analysis

Gel mobility shift analysis at elevated hydrostatic pressure of *Bam*HI binding to a DNA oligonucleotide that contains the cognate recognition sequence reveals that pressure shifts the binding equilibrium favoring dissociation (Fig. 1, A and B). Upon application of modest pressure, 500 bar, the equilibrium dissociation constant ( $K_d$ ) of *Bam*HI for the cognate site is increased almost 10-fold, indicating a loss of binding or transition to a lower-affinity binding state. Over the range of pressures tested, the equilibrium dissociation constants are  $0.7 \pm 0.1$  nM (ambient),  $1.5 \pm 0.1$  nM (150 bar),  $2.5 \pm 0.3$  nM (300 bar), and  $4.6 \pm 0.4$  nM (500 bar). The dependence of dissociation constant on pressure allowed the determination of the volume change ( $\Delta V$ ) of  $-92 \pm 8$  ml/mol

for the dissociation of the specific *Bam*HI-DNA complex (Fig. 1 C). The loss of binding, in principle, can be attributed to increased hydration of the protein-DNA interface or a structural change that does not favor binding.

### MD simulations

One striking difference between the two simulations at ambient and elevated pressure is the behavior of the carboxyl-terminal end of the protein. For the system under ambient pressure, the aforementioned recognition arm remained sequestered in the DNA minor groove and the protein-DNA contacts were maintained throughout the simulation. However, at elevated pressure, the recognition arm became dissociated from the DNA (Fig. 2). Pressure has acted to hydrate, and thus sever, any direct or water-mediated contacts between residues Asp<sup>196</sup>, Gly<sup>197</sup>, Met<sup>198</sup> and DNA, as revealed both through visualization of the trajectories and analysis of the interaction energies between these residues and DNA (Fig. 3).

Inspection of the interaction energies between protein residues and DNA, for both direct and water-mediated con-



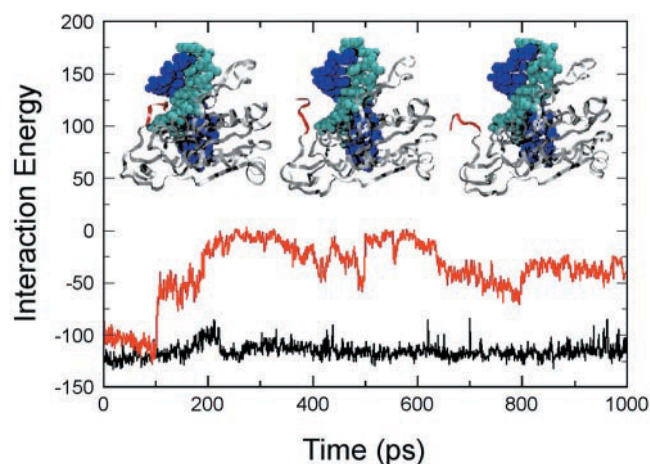


FIGURE 2 The interaction energy between the recognition arm (red) and DNA during both ambient (black line) and elevated pressure (red line) MD simulations. Snapshots of the *Bam*HI R subunit-DNA complex throughout the elevated pressure simulation are displayed above the interaction energy plot. *Left*: snapshot of the equilibrated structure. *Middle* and *right*: sequential snapshots of the complex as the pressure is increased. The interaction energy analysis and the snapshots clearly illustrate that the recognition arm is completely removed from the DNA minor groove as pressure is increased. For clarity, only one monomer of the protein is shown.

tacts, reveals that the direct hydrogen bond between Lys<sup>193</sup> and the phosphate backbone of cytosine (5'-GGATCC-3') is lost early in the simulation. Lys<sup>193</sup> is located at the protein-DNA interface near the arm and is pushed away from the DNA as the arm is displaced from the minor groove. The loss of interaction between the DNA and

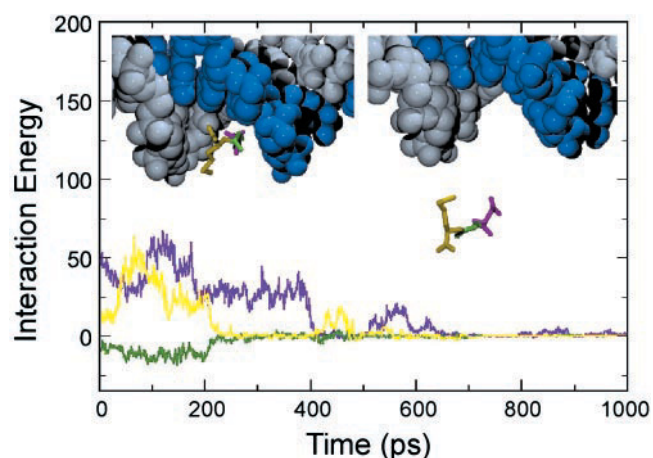
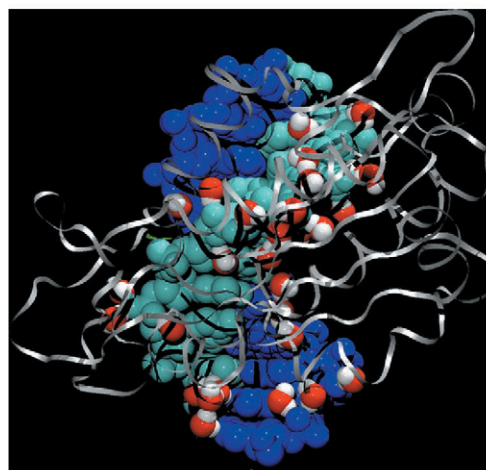


FIGURE 3 Interaction energies between individual residues (Asp<sup>196</sup>-purple, Gly<sup>197</sup>-green, Met<sup>198</sup>-yellow) in the recognition arm and DNA during the elevated pressure simulation. At the beginning of the simulation, these residues are within hydrogen bonding contact with the DNA (*left insert picture*) and as the simulation progresses, the contacts diminish (*right insert picture*) and their interaction energy decreases to zero. The residues are color-coordinated with the interaction energy line it represents in the interaction energy calculation.

A



B

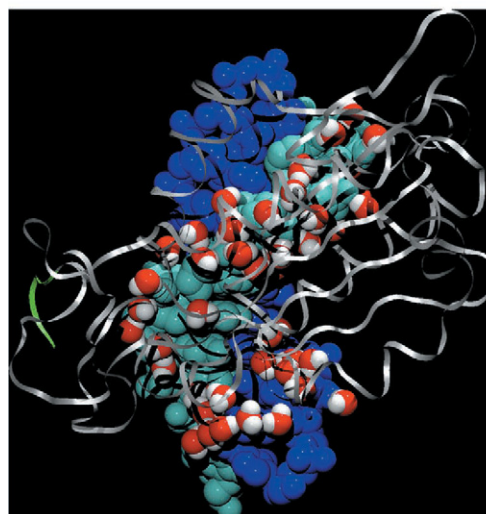


FIGURE 4 Hydration at the *Bam*HI-DNA interface. Snapshots of water molecules at the protein-DNA interface in the MD simulation of the complex at ambient pressure (A) and elevated pressure (B). All water molecules within 3 Å of the protein and the DNA are represented. As observed in the bottom picture, the number of water molecules increases in the simulation at elevated pressure.

(Lys<sup>193</sup>, Asp<sup>196</sup>, Gly<sup>197</sup>, Met<sup>198</sup>) favors dissociation of the specific *Bam*HI-DNA complex.

Along with the loss of interaction between protein residues and DNA, there is another factor that plays a role in the disruption of the specific *Bam*HI-DNA complex. Analysis of interfacial water molecules throughout both simulations reveal that pressure allows a number of water molecules to partition into the protein-DNA interface. It was found that the average number of water molecules at the protein-DNA interface in the ambient pressure simulation remained con-

stant, but increased in the simulation at elevated pressure. A snapshot of interfacial water molecules in both simulations is presented in Fig. 4.

The catalytic site of the enzyme is relatively unaffected by pressure as evidenced by a lack of observable change of the interaction energies between DNA and the catalytic residues Glu<sup>77</sup>, Asp<sup>94</sup>, Glu<sup>111</sup>, and Glu<sup>113</sup> throughout either ambient and elevated pressure simulation. As shown previously, *Bam*HI maintains catalytic activity at the cognate site up to pressures of 1 kbar (Robinson and Sligar, 1995b), and the present simulations are consistent with the suggestion that *Bam*HI achieves specificity part from binding and part from catalysis (Viadu and Aggarwal, 1998). Therefore, the combinations of increased interfacial hydration coupled with loss of specific interactions are most likely the reasons why *Bam*HI-DNA binding equilibria change as pressure is elevated.

## DISCUSSION

Our investigation has led to two important advances. First, this is the first study in which a gel mobility shift assay was used to investigate protein-nucleic acid complex stability at elevated hydrostatic pressure. At the same time, we introduce high-pressure MD simulations on a protein-DNA complex targeted to understanding the binding process and complex stability. High-pressure gel shift analysis and MD simulations demonstrate that the cognate *Bam*HI-DNA complex is disrupted at elevated pressure.

The use of MD simulation was critical in understanding the effects of pressure on the protein-DNA complex at the molecular level. The simulation at elevated pressure clearly illustrates that the recognition arm of *Bam*HI is removed from the minor groove as pressure is increased. We suggest that the key factor involved in the change in binding equilibrium of the specific complex with pressure is the removal of this recognition arm followed by an increased hydration at the protein-DNA interface. The MD analyses therefore provided insight into the mechanism of pressure dissociation in the *Bam*HI-DNA specific complex and offers a clear representation of a less stable complex. The simulation may have also provided an illustration of the structure of *Bam*HI during the initial steps of the dissociative process. MD also reveals that the core structure of the protein is relatively unperturbed by pressure, which is consistent with the previously mentioned fact that *Bam*HI is able to perform site-specific DNA catalysis at elevated pressures.

In summary, the combination of simulation and experimental measurements at elevated hydrostatic pressures offers a means for dissecting the role of direct and water-mediated contacts in protein-nucleic acid complexes.

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