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High Pressure Gel Mobility Shift Analysis and Molecular Dynamics: Investigating Specific Protein-Nucleic Acid Recognition

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In this report, we describe the application of electrophoresis for monitoring protein-DNA binding equilibria at high pressure utilizing the gel mobility shift assay. The first protein-DNA recognition complex studied using this methodology is the restriction endonuclease *Bam*HI binding the cognate DNA recognition sequence. The application of hydrostatic pressure to the specific recognition complex of *Bam*HI-DNA favors dissociation, which is apparent due to the increase in the equilibrium dissociation constant (K_d) at elevated pressures. From the dependence of K_d on pressure, the volume change (ΔV) of dissociation was determined. Molecular Dynamic (MD) simulations on the *Bam*HI-DNA complex at both ambient and elevated pressures were performed to identify the structural origins of the observed experimental results. The simulation trajectories have identified important protein-DNA recognition elements that are disrupted with pressure. The trajectories also illustrate an increased hydration of the *Bam*HI-DNA interface at elevated pressure. Both of these calculated pressure effects would favor dissociation of the complex. The combination of MD simulations and high pressure gel shift analysis proved useful in identifying these factors for maintaining *Bam*HI-DNA complex stability.

1. INTRODUCTION

Previously described experiments using high pressure electrophoresis have demonstrated the usefulness of this technique in biochemical research. Hydrostatic pressure is a convenient and efficient method for perturbing macromolecular equilibrium and the conformation of biological systems. Polyacrylamide gel electrophoresis is an excellent method for separating biomolecules according to size, charge, and conformation. Combining pressure and electrophoresis allows the determination of the stoichiometry of dissociation and the thermodynamic parameters ΔV and K_d. Previous investigations have incorporated high pressure electrophoresis towards understanding oligomeric protein assemblies (1), monomeric

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protein conformation (2), and macromolecular association (3). The method of gel mobility shift analysis at high pressure to study protein-nucleic acid interactions however was never utilized until now.

The gel electrophoresis mobility shift assay is a useful and popular tool for studying protein-nucleic acid interactions. (4,5) The mobility shift assay consists of adding a DNA binding protein to a solution containing a radiolabeled DNA oligomer, harboring a recognition sequence, which can be tightly bound by protein. Following equilibration, the protein-DNA complex is separated from free DNA and/or free protein by electrophoresis. The resolved species are then visualized and their stoichiometries are determined. A flatbed electrophoresis vessel, capable of maintaining hydrostatic pressures up to 2 kbar, was constructed for performing high pressure gel mobility shift assays to monitor the effects of pressure on protein-nucleic acid complexes.

The *Bam*HI 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+}) after the first 5'-G, leaving staggered four base pair overhangs. (6,7) Numerous x-ray structures of the *Bam*HI-DNA complex have been determined that illustrate the interactions involved in site-specific recognition. (8) The factors that contribute to the stability of this complex cannot be identified with structural analysis alone. Therefore, it is necessary to combine structural information with thermodynamic analysis to understand the physical basis for sequence specificity and protein-nucleic acid complex stability. (9) To probe the pressure stability of the specific *Bam*HI-DNA complex we have employed the use of high pressure binding assays.

Molecular Dynamic (MD) simulations are an important theoretical tool and have been used to model the detailed behavior of protein-nucleic acid complexes. (10,11) These calculations offer detailed insight towards understanding the thermodynamic contributions associated with site-specific recognition. To explore the effects of pressure on the cognate *Bam*HI-DNA complex, MD simulations of the complex at both ambient and elevated pressure were performed. The structural origin of the observed pressure effects on the specific *Bam*HI-DNA complex was identified using this analysis and discussed below.

1.1 The specific *Bam*HI-DNA complex

The main feature of *Bam*HI structure is a large six-stranded mixed β-sheet surrounded by α-helices. The x-ray structure of *Bam*HI bound to a DNA oligonucleotide containing the cognate recognition sequence 5'-GGATCC-3' provides insight into site-specific recognition. This complex illustrates a wide range of protein-DNA interactions including side chain atoms, main chain atoms, and bound water molecules. While the DNA does not significantly change form, *Bam*HI undergoes a sequence of conformational changes on complex formation. The most notable structural change is the unraveling of the C-terminal α^7 helices, which are referred to as arms. The arm from the L monomer is positioned along the DNA phosphate backbone towards the *Bam*HI core, while the arm from the R monomer makes specific contacts in the DNA minor groove. The R monomer arm makes direct contacts with DNA through three protein residues, Asp^{196}, Gly^{197}, and Met^{198}. The unraveled arm, which inserts in the minor groove is pictured in Figure 1.

The (R monomer) arm is an important recognition element, as demonstrated by an investigation by Aggarwal and coworkers that intended to dissect the mechanism of DNA strand hydrolysis. (13) In the study, the cognate *Bam*HI-DNA crystal was soaked with Mn^{2+},

to initiate DNA hydrolysis, and cleavage only occurred in the DNA strand bound by the R monomer with the arm situated in the DNA minor groove. (13)

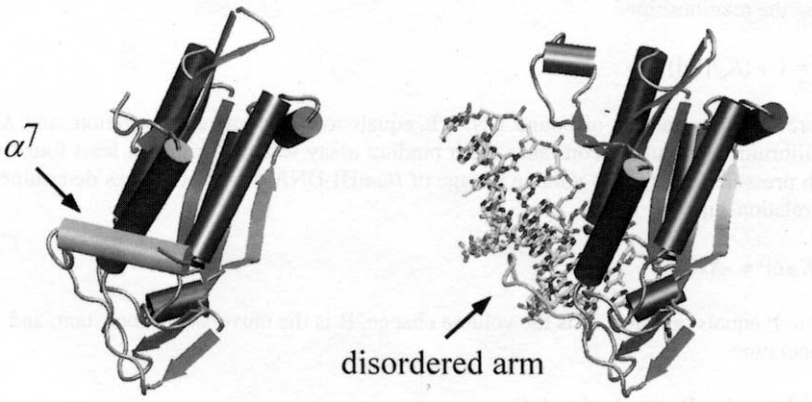

Figure 1. The free (left) and specific bound (right) forms of *Bam*HI. In the free form the C-terminal end exists as helix α^7, when bound to specific DNA this terminus becomes disordered and inserts into the minor groove. The R monomer in which the arm inserts into the minor groove is pictured for clarity.

2. EXPERIMENTAL PROCEDURES

2.1 High pressure gel shift assays

DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and purified separately by polyacrylamide gel electrophoresis. Two complementary sequences (5'-CTCGTATAAT*GGATCC*GCAGTAAGCT-3') containing the *BamHI cognate recognition sequence* were used for binding analysis. The complementary oligonucleotides were 5' end-labeled with [γ-^{32}P]ATP (Amersham) by T_4 polynucleotide kinase (Life Technologies) as previously described (14). The labeled DNA strands were extracted with phenol-chloroform-isoamyl alcohol (24:25:1) and passed through a Bio-Rad P6 spin column to separate the labeled strands from the unincorporated nucleotides. Complementary oligonucleotides were annealed by mixing equimolar concentrations of the DNA strands, heating the mixture to 90° C, and allowing to cool slowly to room temperature. Equilibrium binding assays were performed in the buffer consisting of 10 mM Tris-HCl, 150 mM NaCl, 100 μg/mL bovine serum albumin, 0.1 mM EDTA (pH 8.0), 1 mM dithiothreitol, pH 7.9. Various concentrations of *Bam*HI were incubated at 25° C with the ^{32}P-labeled DNA (1 pM). 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 for 1.5 hours. The assays were run at four pressures: 1) ambient, 2) 150 bar, 3) 300 bar, and 4) 500 bar. Each binding assay was performed at least four times for each pressure tested. Gels were fixed, dried, and exposed using a Molecular Dynamics Phosphorimaging

screen. Band intensities of the complexed and uncomplexed DNA were quantified using ImageQuant software with the volume measurement utility.

The equilibrium dissociation constant (K_d) was determined as described previously (15), using the relationship:

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

where Θ 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 *Bam*HI-DNA dissociation was determined using the relationship:

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

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

2.2 Molecular Dynamic simulations

The protein-DNA system was constructed using the crystal structure of the cognate *Bam*HI-DNA complex (Protein Data Bank entry 1bhm). (8) The DNA contains 11 base pairs with the overhanging 5' Thymine base removed and missing protein residue atoms were modeled using X-PLOR. (16) 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 (dimensions 88Å x 88Å x 88Å) was superimposed on the protein-DNA system and water molecules closer than 2.4 Å to the protein-DNA complex were removed. Thirty-two sodium ions were added to the system, by replacing water molecules, to bring the resulting protein-DNA-solvent system to charge neutrality. The system contains approximately 65,300 atoms. After equilibration, the system was simulated under ambient and elevated pressure for one nanosecond (ns) at 297 K. The MD simulations were performed using the program NAMD2 (17), with v.26 of the CHARMM force field (18). All hydrogen bonds were constrained using SHAKE and a time step of 2 femtoseconds (fs) was used. The system was simulated using Langevin dynamics in an NpT ensemble with periodic boundary conditions and full electrostatics were calculated using the particle mesh Ewald (PME) method. (19) For the simulation at elevated pressure, a gradient of 50 bar/100 picoseconds (ps) was used until a pressure of 400 bar was attained. Constant pressure was maintained using the Langevin piston method (20) with a piston period of 200 fs and damping time constant of 100 fs. Interfacial water molecules are defined as those molecules that are 3 Å from both the protein and DNA throughout the simulation. Simulation snapshots were created using the molecular graphics program VMD (http://www.ks.uiuc.edu).

3. RESULTS ANS DISCUSSION

3.1 High pressure gel mobility shift analysis

The system we have chosen for our initial study is the specific *Bam*HI-DNA complex. The specificity and stability of this complex has previously been shown to be sensitive to pressure

(21), making it an ideal system for the first high pressure gel shift analysis. The application of hydrostatic pressure during the gel shift analysis of *Bam*HI causes a shift in the equilibrium dissociation constant for the cognate site. After measurement of the dissociation constant at several elevated pressures the volume change (ΔV) associated with *Bam*HI dissociation was also determined using. (Figure 2)

Gel mobility shift analysis of *Bam*HI binding to a specific DNA oligonucleotide, pictured in Figure 2, clearly illustrates that elevated hydrostatic pressure shifts the binding equilibria towards a lower affinity binding state.

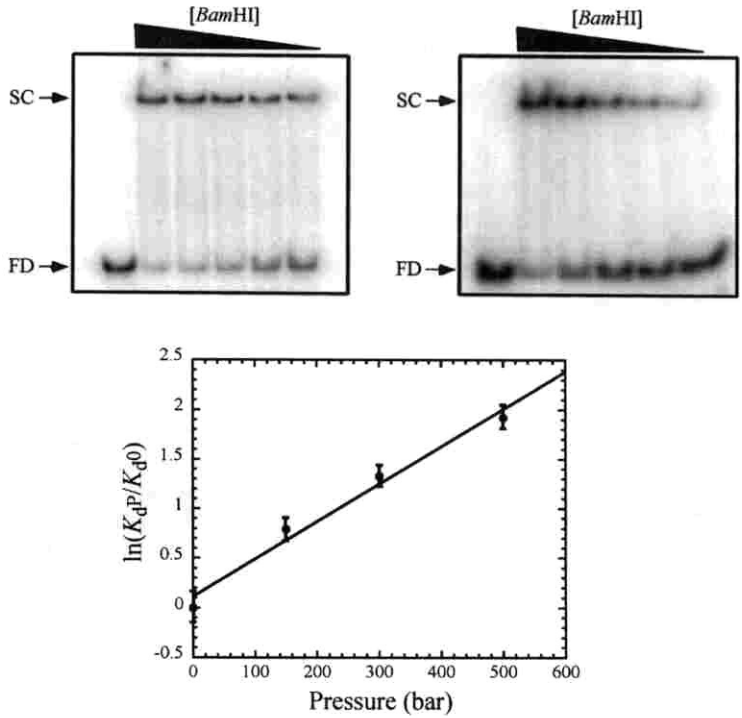

Figure 2. Gel mobility shift analysis at ambient pressure (top left) and 300 bar elevated pressure (top right). An identical protein titration was used for both illustrated binding assays. **SC** – specific *Bam*HI-DNA complex, **FD** – free DNA probe. The dependence of dissociation constant (K_d) on hydrostatic pressure (bottom).

The measured equilibrium dissociation constants (K_d), over the range of pressures tested, are 0.7 ± 0.1 nM (ambient), 1.5 ± 0.1 nM (150 bar), 2.5 ± 0.3 nM (300 bar), 4.6 ± 0.4 nM (500 bar). The dependence of dissociation constant on pressure, plotted in Figure 2, revealed a volume change (ΔV) of -92 ± 8 mL/mol for the pressure induced dissociation of the specific *Bam*HI-DNA complex. The loss of binding may be due to a structural change that decreases

the overall volume of the system that favors dissociation, or an increased hydration of the protein-DNA interface that could disrupt specific protein-DNA interactions.

3.2 Molecular Dynamic simulation trajectories

The MD simulation calculations were performed to provide insight in to the observed high pressure effects during the binding analysis. The first trajectory calculated was the cognate *Bam*HI-DNA-solvent complex at ambient pressure; the second was the complex being exposed to a pressure gradient. We have compared the two simulations both visually and through interaction energy analysis to determine the effects of pressure at the molecular level. The previously identified direct and water-mediated protein-DNA contacts in the cognate *Bam*HI-DNA complex (21) were monitored during both trajectories. The measurement of protein-DNA interaction energies offers detailed analysis that may have been overlooked during the initial screening of the trajectories.

In viewing the trajectories of both systems, the most noticeable effects of pressure are manifested in the removal of the (R monomer) arm from the DNA minor groove. Interaction energy analysis between the arm residues (Asp^{196}, Gly^{197}, and Met^{198}) and DNA throughout both simulations confirms that the application of pressure disrupts these specific interactions. The direct and water-mediated contacts are maintained during ambient pressure simulation. Figure 3 illustrates the dissociated arm observed in the elevated pressure simulation versus the intact arm, which remains sequestered in the minor groove during the ambient pressure simulation.

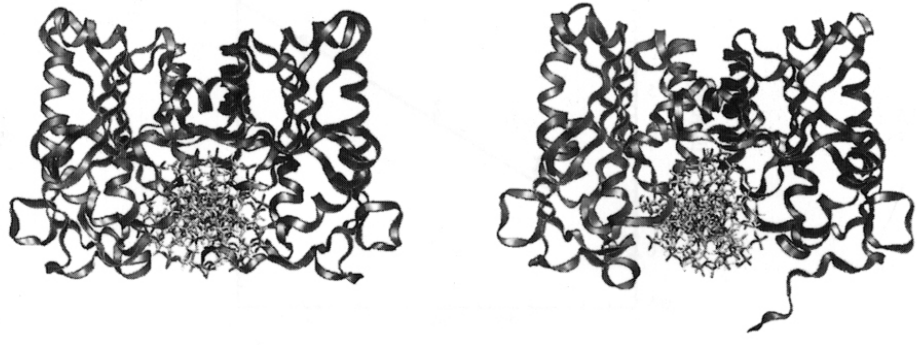

Figure 3. MD simulation snapshots of the equilibrated specific *Bam*HI-DNA complex (left) and the complex at an elevated pressure of 400 bar (right). At elevated pressure, the arm becomes dissociated from the DNA minor groove (right) and contacts near the protein-DNA interface are apparently disrupted.

The detailed interaction energy analysis reveals that the contact between Lys^{193} and the DNA phosphate backbone is lost at elevated pressure. This loss of interaction was not identified in the initial trajectory screening phase. The simulation at elevated pressure illustrates that Lys193 is no longer within hydrogen bonding distance in the pressure equilibrated state of the *Bam*HI-DNA complex.

Endonuclease function relies on both enzyme specificity and the ability to catalyze DNA strand hydrolysis. A part of the interaction energy analysis focused on the effects of pressure

on the active site residues Glu^{77}, Asp^{94}, Glu^{111}, and Glu^{113}. *Bam*HI has been shown previously to maintain catalytic activity at pressure up to 1.2 kbar. (22) The interaction energy analysis confirms that pressure does not affect the interactions between DNA and the catalytic residues, which is consistent with the aforementioned experimental observations.

The effect of pressure-induced hydration was probed by determining the number of water molecules associated with the *Bam*HI-DNA interface during both simulations. The simulations reveal that a number of water molecules enter the protein-DNA interface at elevated pressure, which are absent at ambient pressure. The water molecules, illustrated in Figure 4, are those that reside at the *Bam*HI-DNA interface in the equilibrated states of the complex at ambient and elevated pressure (400 bar).

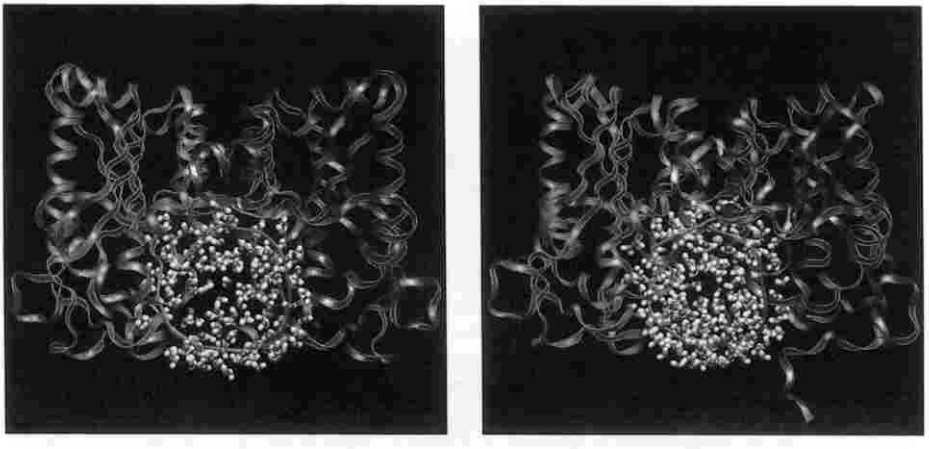

Figure 4. MD simulation snapshots of all water molecules within 3 Å of both the protein and DNA at both ambient pressure (left) and 400 bar (right). Water molecules are highlighted in white.

5. CONCLUSIONS

In this report, we have examined the pressure dependence of the interaction between the restriction endonuclease *Bam*HI and its cognate DNA recognition sequence. Application of hydrostatic pressure favors the dissociation of the specific complex indicating that the unliganded state of *Bam*HI displays a smaller molecular volume than that of the bound state (ΔV = -92 ± 8 mL/mol). To examine the possible structural origins of the observed volume change we have performed MD simulations on the complex at elevated pressure. The MD simulation trajectories illustrate two key features that give rise to the loss of binding affinity. First, the recognition arm is displaced from the minor groove and second, there is an observed increase in hydration at the protein-nucleic acid interface. Both of these structural changes are consistent with the observation of a negative volume change. Removal of the arm exposes the charges of the DNA that would result in reordering of the solvent in the minor groove and

around the exposed recognition arm. These results demonstrate the importance of combining theory and experiment to gain insight into thermodynamic equilibria and identify key factors involved in specific protein-nucleic acid recognition.

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