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Hydrostatic Pressure Reverses Osmotic Pressure Effects on the Specificity of *Eco*RI–DNA Interactions†

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ABSTRACT: To characterize the role of water in protein–DNA interactions, we have studied the specificity of the *Eco*RI restriction endonuclease as a function of osmotic and hydrostatic pressure. The extent of cleavage by the enzyme at noncanonical ("star") sites is shown to depend uniquely upon the osmotic pressure in the reaction as controlled by the addition of a wide variety of neutral solutes. Alteration of cleavage specificity ("*Eco*RI* activity") is not uniformly correlated with any other colligative solvent property such as dielectric constant, viscosity, or water concentration. The application of hydrostatic pressure reverses the effects of osmotic pressure, restoring the natural selectivity of the enzyme for its canonical site GAATTC. This combination of observations provides compelling evidence that the site-specific recognition of canonical site DNA by *Eco*RI is mediated by discretely bound water molecules and that the release of these waters induces a fundamental change in the specificity of the interaction, leading to cleavage at alternative sites. This comprehensive analysis of solvent effects facilitates the unambiguous identification of structurally and functionally specific waters involved in macromolecular recognition events.

Protein–DNA interactions are crucial for many biological process and present a unique challenge both for understanding binding specificity and for the design of novel therapeutics and research tools. Restriction enzymes represent superb examples of recognition efficiency and accuracy in DNA cleavage and serve as an ideal system for the study of site-specific protein–DNA interactions (Fisher & Gumport, 1994). In particular, the *Eco*RI restriction endonuclease is one of the most well-studied enzyme systems and serves as a paradigm for sequence-specific protein–DNA interactions and for molecular recognition events in general (Freemont et al., 1991; Rosenberg, 1991). *Eco*RI is a homodimer of 30-kDa monomers which cleaves the DNA sequence GAATTC on both strands between G and A (Heitman, 1992). The structure of *Eco*RI complexed with an oligodeoxyribonucleotide containing this target recognition sequence has been solved at high resolution by X-ray crystallography (Rosenberg, 1991). Binding constants and catalytic rate constants have been established for all single base substitutions of the canonical sequence (Lesser et al., 1990; Thielking et al., 1990). The enzyme achieves both tight binding and extremely high accuracy by means of an intricate hydrogen-bonding network to the target DNA sequence. However, under certain conditions, the specificity of the enzyme is reduced, and cleavage can occur more readily at alternative sites containing one noncanonical base pair (Gardner et al., 1982; Malyguine et al., 1980; Polisky et al., 1975; Rosenberg & Greene, 1982). This relaxation of specificity has been termed *Eco*RI* ("star") activity. Recently, we have shown a striking correlation between the osmotic pressure of the reaction and the extent of star activity (Robinson & Sligar, 1993). This correlation implicates the participation of water in the site-specific recognition of DNA by the enzyme.

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The importance of interstitial water molecules in mediating specificity of protein–DNA interactions has been clearly demonstrated by structural studies, for example, in the binding of the *trp* repressor to its target operator sequence (Carey et al., 1991; Lawson & Carey, 1993; Otwinowski et al., 1988). Additional examples of water-mediated contacts with DNA include the *lac* repressor/operator complex (Ha et al., 1992) and Hoechst compounds 33258 and (+)-CC-1065 (Lin et al., 1991).

Recently, a number of investigators have further examined the roles of water in macromolecular interaction, stability, and function. Bound water has been shown to participate in oxygen binding to hemoglobin (Colombo et al., 1992), electron transfer in cytochrome *c* oxidase (Kornblatt & Hui Bon Hoa, 1990), glucose binding to hexokinase (Rand et al., 1993), stabilization of lysozyme, ribonuclease, and other proteins (Arakawa & Timasheff, 1982; Santoro et al., 1992), binding of cytochrome *c* to cytochrome *c* oxidase (Kornblatt et al., 1993a), the catalytic cycle of cytochrome P-450 (Di Primo et al., 1992), and formation of an active "hydrated" form of adenosine deaminase (Dzingeleski & Wolfenden, 1993).

In many cases these proposals that discretely bound water molecules play an integral part in the recognition process utilize application of osmotic stress to perturb protein conformation or binding equilibria. Since osmotic pressure (π) controls the activity of water in an aqueous compartment inaccessible to neutral solutes (osmolytes), osmotic stress induces the release of bound water from macromolecules to bulk solvent. Macromolecular conformations are thus shifted toward the state with the least bound water. Therefore, in the simplest case, a correlation between osmotic pressure and a biomolecular process, such as ligand binding, is commonly interpreted as evidence for a role of bound water in the process (Rand, 1992).

The application of hydrostatic pressure has also been widely used to investigage protein–protein interactions (Weber, 1992). The direct effect of hydrostatic pressure—to shift equilibria to the state of lowest overall volume—typically results in enhanced solvation of macromolecular systems, due to the differential compressibilities of water–water, water–protein, and protein–protein contacts (Weber, 1993). Accordingly, hydrostatic pressure tends to dissociate oligomeric protein complexes (1–2 kbar) and induce protein unfolding (>4 kbar). One outcome of this phenomenon is the prediction that hydrostatic pressure should compensate for the effects of osmotic pressure upon the state of bound water molecules, as observed in formation of cytochrome b_5–cytochrome *c* complexes (Kornblatt et al., 1993a; Rodgers et al., 1988) and enolase dimers (Kornblatt et al., 1993b).

Previously, we have shown that the specificity of *Eco*RI, as measured by the fraction of cleavage at noncanonical (star) sites in DNA, is correlated with osmotic pressure induced by ethanol, dimethyl sulfoxide, glycerol, ethylene glycol, sucrose, and dextrose (Robinson & Sligar, 1993). We report here an analysis of *Eco*RI specificity as a function of hydrostatic pressure. Hydrostatic pressure is found to reverse the effects of osmotic pressure upon *Eco*RI specificity, providing conclusive evidence that bound water participates in determining the sequence specificity of *Eco*RI DNA cleavage. By examining dielectric constant, viscosity, and water molarity, we also demonstrate that a correlation with the effects upon *Eco*RI specificity is unique to osmotic pressure—no other solvent property displays a uniform correlation with star activity for all compounds tested. Furthermore, through kinetic analysis, we show that the effect of osmotic pressure is manifested at least in part as an altered selectivity of the enzyme, rather than a simple relaxation in specificity.

The approach described here couples the use of hydrostatic pressure with an analysis of the relationship between observed phenomena and numerous solvent properties over a wide range of neutral solutes and cosolvents. Such a strategy provides a generally applicable procedure to develop an accurate model for the role of solvent in mediating macromolecular interactions.

EXPERIMENTAL PROCEDURES

Reaction conditions were essentially as described previously (Robinson & Sligar, 1993). Enzymatic reactions at ambient pressure (shown in Figure 1) contained 10 ng/μL (6.2 nM) pUC18 DNA (purified using Promega Magic MegaPreps) and 1 unit/μL (5.5 nM) *Eco*RI (Gibco/BRL). 2-Propanol, *N*-methylformamide, sucrose, and glycerol were added to final concentrations from 0 to 4 M to induce osmotic pressures from 0 to 100 atm. Values for osmotic pressure were calculated (Tombs & Peacocke, 1974) both from measured osmolalities using a UIC 070 vapor-phase osmometer and from tabulated data (Wolf et al., 1986–1987). Viscosities, dielectric constants, and water molarity were taken from published tables (Dean, 1985; Wolf et al., 1986–1987).

The pUC18 plasmid is 2686 base pairs long and contains one *Eco*RI site (GAATTC) at position 450. According to the detailed hierarchies of *Eco*RI star activity (Lesser et al., 1990; Rosenberg & Greene, 1982), pUC18 also contains two readily cleaved *Eco*RI* sites: GAGTTC at position 1267 and TAATTC at position 2127 (noncanonical bases in boldface). Under the reaction conditions in the experiments performed, five different bands were observed. Cleavage by *Eco*RI at the canonical site produces a linear strand of pUC18. Subsequent cleavage at the star yields fragments with sizes ranging from 817 to 1869 base pairs. The bands resulting from star activity can be easily distinguished from the singly digested plasmid (Figure 2A).

Samples were analyzed as described previously (Robinson & Sligar, 1993). Briefly, products of the reactions were separated by slab agarose gel electrophoresis and visualized by staining with ethidium bromide. The gels were photographed, scanned, and then analyzed using Image 1.44 (Wayne Rasband, NIH) to measure relative band intensities. Intensities of bands from serial dilutions of DNA samples were quantified to ensure that all measurements were performed within the linear response range of the film and camera. "Fraction star activity" was defined as the combined intensities of the bands resulting from cleavage at the star sites divided by the total intensity of all bands.

Reactions at elevated hydrostatic pressure were performed in 250-μL polypropylene tubes (Cole Parmer), incubated in a high-pressure reactor (HiP Co., Erie, PA), with pressure generated by a manual pump. Glycerol was used to induce osmotic pressure from 0 to 100 atm. To allow the reaction to be initiated only after application of hydrostatic pressure, 150 μL of enzyme solution was separated from 70 μL of DNA solution by a 75-μL layer of molecular biology grade mineral oil (Sigma). For each reaction, 0.2 g of zirconium oxide beads (BioSpec Products, Bartlesville, OK) was placed at the bottom of the tube. After assembly of the tubes in brass inserts in the high-pressure reactor, the vessel was inverted several times to initiate the reaction, as the beads broke the oil layer and allowed the enzyme and DNA solutions to mix freely. The samples were incubated at the desired pressure for 4 h at 37 °C, followed by a 30-min incubation at 65 °C to inactivate the enzyme. After release of pressure and removal of the

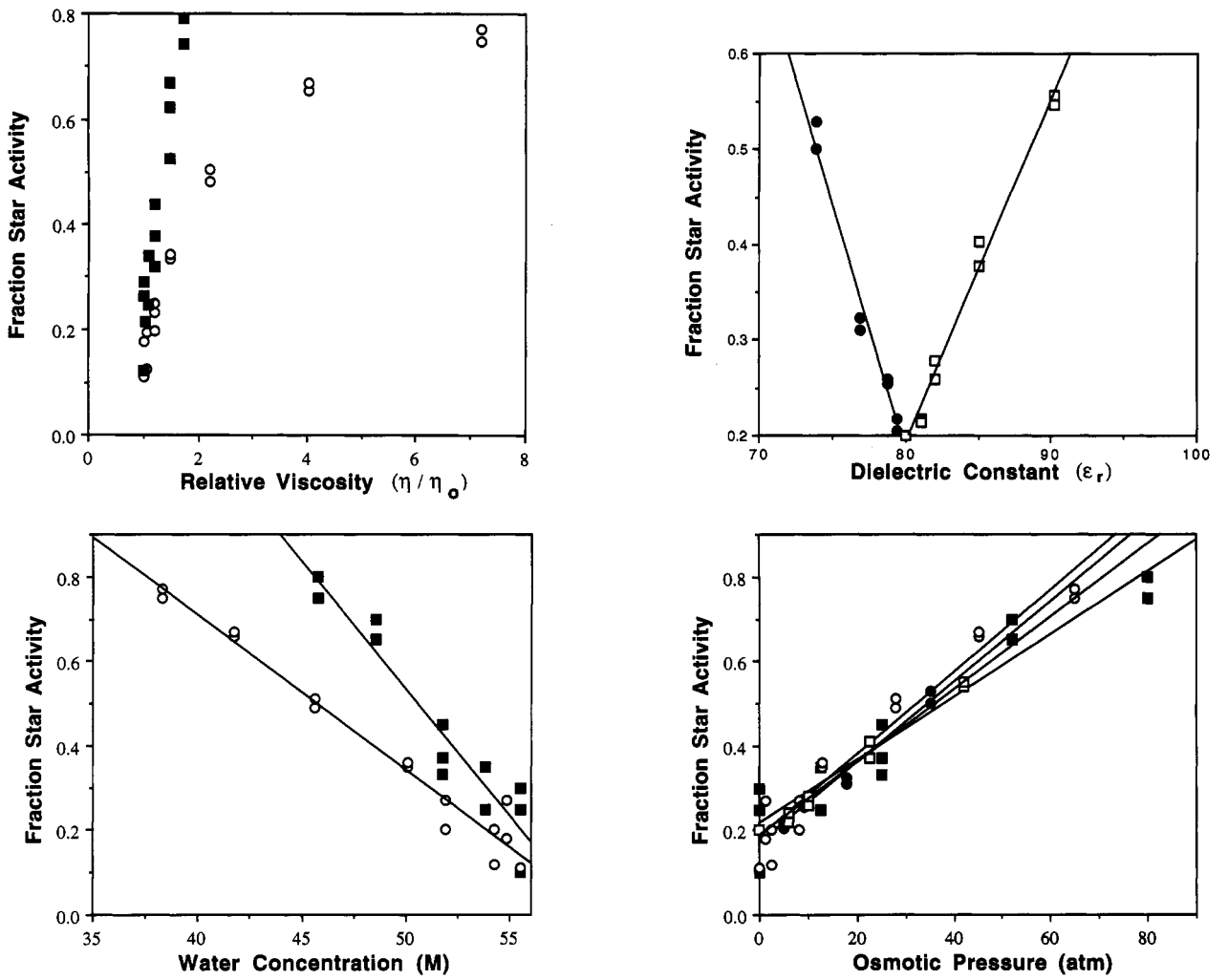

FIGURE 1: Fraction of *Eco*RI* activity correlates uniformly only with osmotic pressure and not with relative viscosity, dielectric constant, or water concentration. Increasing concentrations of the four reagents used induced increases in the intensity of bands corresponding to cleavage at star sites. The fraction of *Eco*RI* activity is plotted as a function of solvent properties at increasing concentrations of sucrose (○), glycerol (■), 2-propanol (●), and *N*-methylformamide (□). (A, top left) Star activity versus relative viscosity using sucrose and glycerol. (B, top right) Star activity versus dielectric constant using 2-propanol and *N*-methylformamide. (C, bottom left) Star activity versus water molarity for sucrose and glycerol. (D, bottom right) Star activity versus osmotic pressure for sucrose, glycerol, 2-propanol, and *N*-methylformamide. Straight lines indicate the best fit by the least squares analysis.

reaction tubes from the high-pressure chamber, EDTA was added to the samples to a final concentration of 100 mM to ensure that no further reaction would occur.

To eliminate possible effects of competition between the two sites, the kinetic analysis was performed using individual DNA fragments of pUC18 containing single canonical or star sites. These fragments were generated by restriction digests, separated by agarose gel electrophoresis, and purified using Magic PCR preps (Promega). Accordingly, reactions containing either one canonical *Eco*RI site (GAATTC) *or* one star site (TAATTC) were performed independently. Samples of *Eco*RI and DNA were incubated for 2 h at 37 °C in magnesium-free buffer of 25 mM Tris-HCl, pH 8.0, containing glycerol to induce osmotic pressures of 0, 50, and 100 atm. Reactions were initiated by addition of magnesium chloride to a final concentration of 8 mM. Aliquots were removed at the time points indicated and quenched with 100 mM EDTA and 4 M urea.

RESULTS

Of four solution properties—osmotic pressure, viscosity, dielectric constant, and water molarity—only osmotic pressure displays a uniform correlation with the star activity of *Eco*RI (Figure 1). Changes in viscosity yield different increases in the fraction star activity when glycerol or sucrose is used as a cosolvent (Figure 1A). *N*-Methylformamide shows a positive correlation between dielectric constant and fraction star activity, while 2-propanol shows a negative correlation, indicating that dielectric constant does not control recognition specificity (Figure 1B).

Similarly, identical decreases in water molarity do not produce the same increase in fraction star activity for the four compounds, indicating that water concentration is not the operative parameter in *Eco*RI–DNA recognition. At dilute concentrations, glycerol and sucrose display similar correlations between water molarity and star activity, but as water molarity decreases to low levels, this plot diverges for the two compounds (Figure 1C).

The uniform relationship between osmotic pressure and star activity is maintained even at the highest osmotic pressures measured (Figure 1D), consistent with previous results (Robinson & Sligar, 1993). Again, the diversity of the compounds used to control osmotic pressure suggests that there is no direct effect of binding of the osmolytes to the protein–DNA complex to influence specificity.

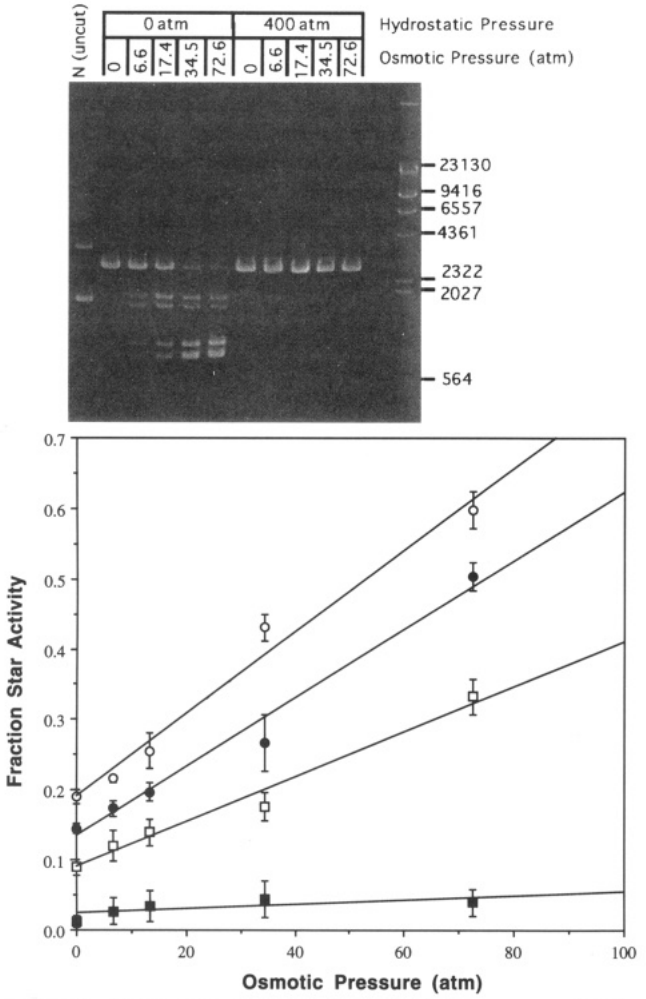

FIGURE 2: Hydrostatic pressure restores *Eco*RI cleavage specificity, reversing the increase in star activity induced by osmotic pressure. (A, top) Agarose gel analysis of the pattern of *Eco*RI cleavage of supercoiled pUC18 DNA. The leftmost lane contains uncut pUC18 DNA; the rightmost lane contains DNA size markers (λ DNA *Hin*dIII digest, Gibco/BRL). The 10 center lanes contain pUC18 DNA incubated with *Eco*RI in the presence of increasing osmotic pressures at 0 and 400 atm of hydrostatic pressure as indicated at the top of the gel. In these lanes, the top band corresponds to the linearized pUC18 which has been cut at only the canonical *Eco*RI site. After cleavage at the canonical site, cleavage of the linearized plasmid at the *Eco*RI* sites yields the four lower bands which correspond to two pairs of fragments: 1869 and 817 bases (resulting from cleavage of GAGTTC at position 1267) and 1677 and 1009 bases (resulting from cleavage of TAATTC at position 2127). This allows the extent of *Eco*RI* activity to be evaluated under each set of buffer conditions. The band corresponding to the full-length linear fragment of pUC18 was cut from the gel and subjected to restriction analysis to confirm that cleavage had occurred only at the canonical site. (B, bottom) Fraction star activity versus osmotic pressure at varied levels of applied hydrostatic pressure. Reactions were performed at the osmotic and hydrostatic pressures indicated. Points represent the average of three trials, and error bars indicate the standard deviation. Straight lines indicate the best fit by least squares analysis. Applied hydrostatic pressures are as follows: ambient pressure (○), 100 atm (●), 200 atm (□), and 400 atm (■).

At a constant level of osmotic pressure, increasing hydrostatic pressures lower, and ultimately eliminate, the fraction of star activity observed. Over the full range of osmotic pressure used, we find a dramatic decrease in the intensity of the bands arising from cleavage at star sites, corresponding to a progressive inhibition of star activity with increasing hydrostatic pressure (Figure 2A). A linear relationship between osmotic pressure and fraction star activity is maintained, but with a decreasing slope as increasing levels of hydrostatic pressure are applied (Figure 2B). The extent of cleavage at the canonical site GAATTC was unchanged between ambient and 400-atm hydrostatic pressure (see caption to Figure 2).

Due to the composition of the buffer system used in these studies, a low level of *Eco*RI* activity occurs even in the absence of additional osmotic pressure. The decrease in fraction star activity at 0-atm added osmotic pressure (Figure 2B) indicates that hydrostatic pressure serves to inhibit this background star activity as well as the more dramatic levels induced by the presence of increasing concentrations of osmolytes.

Interestingly, 400 atm of hydrostatic pressure is required to counteract the effects of 100 atm of osmotic pressure. Presumably, volume changes besides those resulting from resolvation of the complex are induced by hydrostatic pressure. This inequality in the effects of hydrostatic and osmotic pressure reflects the difference between the direct effect of hydrostatic pressure upon volume versus that of osmotic pressure upon water activity (Narayan et al., 1990).

*Eco*RI activity was previously shown to be inhibited by hydrostatic pressures of 2000 atm (MacGregor, 1990). This was interpreted as evidence of pressure-induced dissociation of the protein–DNA complex. However, in our studies we find that relatively low hydrostatic pressures (less than 500 atm) do not inhibit the formation of productive *Eco*RI–DNA complexes. The extent of cleavage at the canonical site is not measurably reduced by the application of these levels of hydrostatic pressure. Only the extent of star activity is affected.

To ascertain the effects upon overall cleavage rate at each site, we measured the extent of cleavage versus time for preformed complexes of *Eco*RI and DNA fragments containing one canonical or one star site at 0, 50, and 100 atm of osmotic pressure. Unexpectedly, the relative rate of cleavage at the star site TAATTC is increased by osmotic pressure, while the cleavage rate at the canonical site GAATTC is decreased (Figure 3). These experiments were performed by first incubating the enzyme with DNA to establish binding equilibrium and then initiating the reaction by addition of magnesium. Therefore, we believe that the results reflect an alteration in the catalytic rate constants for cleavage at the two sites.

DISCUSSION

The addition of "neutral" solutes or cosolvents to a solution induces changes in osmotic pressure and many other solvent properties, which can all affect protein conformation, function, and binding interactions. These include viscosity, dielectric constant, and water molarity (Beece et al., 1980; Dzingeleski & Wolfenden, 1993). A given compound is likely to induce similar correlations between an observable and many colligative properties. In order to identify which aspect is most directly related to the change in function being analyzed, it is necessary to examine the consequences of a variety of compounds with differing effects upon viscosity, dielectric constant, osmotic pressure, and water concentration.

As the concentration of each compound is increased, the fraction *Eco*RI star activity increases as well. As expected for any colligative property, 2-propanol, *N*-methylformamide, glycerol, and sucrose each display correlations between fraction star activity and viscosity, dielectric constant, water molarity, and osmotic pressure. However, the correlations are uniform only for osmotic pressure (Figure 1). That is, the fraction star activity depends only upon osmotic pressure and not upon any other solvent property. Furthermore, since the compounds

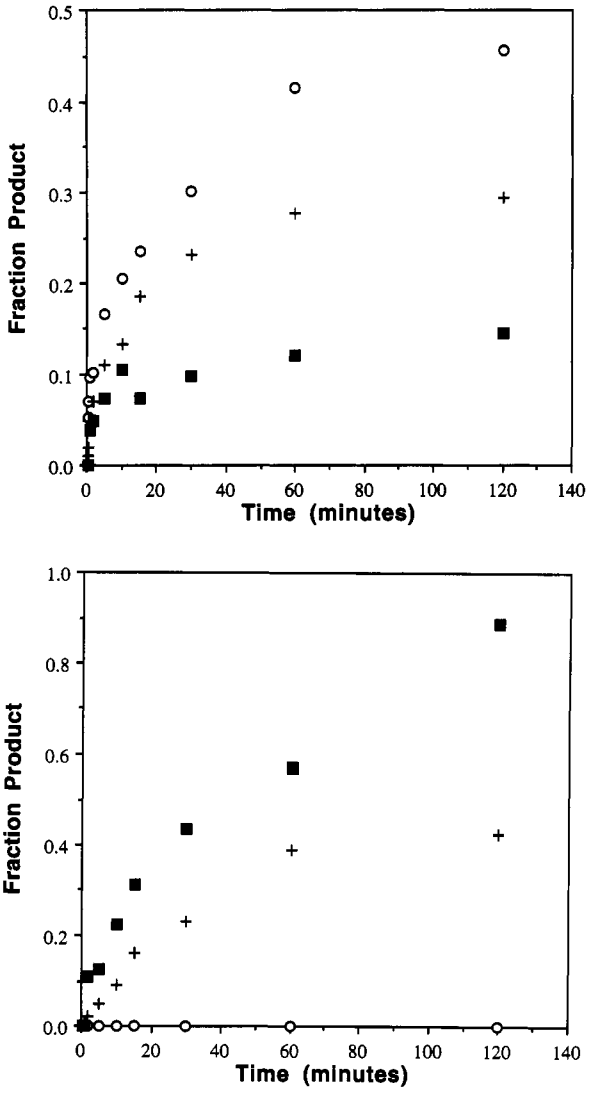

FIGURE 3: Osmotic pressure induces a change in the selectivity of *Eco*RI, causing a decrease in cleavage rate at the canonical site and an increase in cleavage rate for the star site TAATTC. The plots show cleavage of DNA fragments containing single canonical or star sites as a function of time at 0 (○), 50 (+), and 100 atm (■) of osmotic pressure. Panels: (A, top) canonical site (GAATTC) cleavage; (B, bottom) star site (TAATTC) cleavage.

differ widely in their sizes and chemical properties, specific binding between the compounds and the protein, DNA, or the complex, leading to increase in star activity, can be essentially ruled out.

Application of osmotic pressure relies upon the existence of a water-permeable barrier (such as a protein cleft or interface) separating a population of water molecules from a nonpermeating solute (osmolyte). Correlation of a process with osmotic pressure implies a change in the size of this population. Correlation with water molarity, as observed for the activity of calf intestinal adenosine deaminase (Dzingeleski & Wolfenden, 1993), suggests that water, acting as a ligand, binds to the enzyme complex at sites freely accessible to both solvent and solutes.

Our observations that the fraction of *Eco*RI* activity correlates uniquely and uniformly with osmotic pressure indicate that there exist water(s) which are inaccessible to the solutes or cosolvents (osmolytes) studied, which control the specificity of the *Eco*RI endonuclease. The release of these waters leads to the altered recognition which is manifested as *Eco*RI* activity.

Incubation of the enzymatic reactions at elevated hydrostatic pressures reverses the effects of osmotic pressure (Figure 2). Compared to ambient pressure, reactions carried out at hydrostatic pressures of 100 and 200 atm yield cleavage patterns showing a marked decrease in the fraction star activity. At 400 atm, star activity is reduced to undetectable levels. Hydrostatic pressure has a well-established effect of enhancing the solvation of biological macromolecules such as proteins and DNA (Weber & Drickamer, 1983). Accordingly, we interpret our results as conclusive evidence that water molecules released from the *Eco*RI–DNA complex by the application of osmotic pressure are responsible for the increase in cleavage at alternative sites. When hydrostatic pressure is applied, the complex is resolvated, restoring the natural selectivity of *Eco*RI.

The number and location of these waters is the subject of our current investigations. *Eco*RI binds to DNA almost exclusively through contacts in the major groove. Accordingly, structurally and functionally significant waters may be sequestered in the interface of the complex, in the minor groove of DNA, or in clefts on the surface of the protein. Future studies will address the relative binding constants and single- and double-stranded cleavage rates of *Eco*RI for canonical and star sites as a function of osmotic pressure. Our preliminary analysis of the binding and rate constants indicates that bound water participates in sequence-specific recognition in both the association and catalytic steps of the reaction.

One candidate for involvement in this recognition motif is the resident water in the cocrystal structure of the *Eco*RI–DNA complex. In each of the two symmetric sets of contacts between *Eco*RI and DNA, this water molecule is involved in bridged hydrogen bonds between arginine 200 and arginine 203 in *Eco*RI and the guanine base in the recognition sequence (Heitman, 1992; Rosenberg, 1991). Increasing osmotic pressure would serve to draw this water out of the protein–DNA interface. The loss of this water-mediated contact between enzyme and substrate may be responsible in part for the observed loss of specificity. However, this scheme is only a partial explanation, as it does not account for the loss of specificity at other positions. Additional waters may be released from the enzyme, DNA, or the complex by application of osmotic pressure and restored by hydrostatic pressure.

*Eco*RI induces a substantial bend in the DNA helix upon binding (Rosenberg, 1991). Since DNA conformation and flexibility, which are very sensitive to solvent conditions, have been shown to influence protein–DNA recognition (Lesser et al., 1993), it is possible that differential solvation of the DNA due to changes in osmotic and hydrostatic pressures may result in changes in conformation which alter the susceptibility to cleavage or bending at canonical and star sites. Dramatic effects upon DNA deformability have been observed for changes in the minor groove, both from methylation (Kimura et al., 1989; Terry & Modrich, 1983) and from changes in the proposed "spine of hydration" (Liepinsh et al., 1992).

A second class of changes in solvent composition, which alter ionic strength, pH, and divalent cation identity, have also been demonstrated to induce star activity in *Eco*RI and other restriction enzymes (Vermote & Halford, 1992; Woodhead et al., 1981). The effects upon molecular recognition observed when these conditions are used to alter cleavage specificity are not simply correlated with osmotic pressure and may proceed by additional mechanisms, such as those of electrostatic origin.

To test possible mechanisms for the observed correlation between osmotic pressure and star activity, the effect of osmotic

pressure upon cleavage at canonical and star sites was compared. Cleavage of canonical and noncanonical DNA was measured in separate reactions to eliminate effects of competition between the two sites. Surprisingly, cleavage at the canonical site (GAATTC) is diminished at elevated osmotic pressures, whereas cleavage at the star site (TAATTC) is dramatically enhanced (Figure 3). The simplest explanation consistent with these observations and the others presented here is that the specificity of *Eco*RI is *altered* by the release of bound water from the complex.

On the basis of these results, it is unlikely that dehydration of the protein and DNA leads simply to enhanced binding at all DNA sites, making cleavage at "star sites" more frequent. Furthermore, the proposed mechanism for *Eco*RI–DNA recognition involves a loose binding to nonspecific DNA, followed by a translation or "sliding" along the DNA helix until the recognition site is encountered (Terry et al., 1985). Accordingly, an increase in nonspecific binding, either from dehydration or from a reduced effective free volume, does not appear to be sufficient to cause a loss or alteration in specificity observed in these and other studies.

Appreciation for the role of water in biomolecular structure and function continues to expand (Otting et al., 1991; Rand, 1992; Westhoff, 1988; Wolfenden, 1983). In conjunction with structural determinations and analysis of changes in heat capacity (Jin et al., 1993; Spolar et al., 1992), osmotic and hydrostatic pressure techniques represent an important set of tools for studying these phenomena.

We have presented a generally applicable method for the unambiguous identification and characterization of the role of bound waters in macromolecular interactions and recognition events. This approach is based upon a comprehensive analysis of solvent properties perturbed by the addition of a wide range of solutes, coupled with the use of hydrostatic pressure techniques. This method has been used to demonstrate the existence of a number of bound water molecules associated with the *Eco*RI–DNA complex. Release of these waters induces a fundamental change in the selectivity of the enzyme leading to enhanced cleavage at sites other than the canonical GAATTC sequence (*Eco*RI* activity). We anticipate that this approach will be useful to more fully explore the role of bound water in protein–nucleic acid interactions and other biochemical systems.

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