Differences in Hydration Coupled to Specific and Nonspecific Competitive Binding and to Specific DNA Binding of the Restriction Endonuclease BamHI*

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Using the osmotic stress technique together with a self-cleavage assay we measure directly differences in sequestered water between specific and nonspecific DNA-BamHI complexes as well as the numbers of water molecules released coupled to specific complex formation. The difference between specific and nonspecific binding free energy of the BamHI scales linearly with solute osmolal concentration for seven neutral solutes used to set water activity. The observed osmotic dependence indicates that the nonspecific DNA-BamHI complex sequesters some 120–150 more water molecules than the specific complex. The weak sensitivity of the difference in number of waters to the solute identity suggests that these waters are sterically inaccessible to solutes. This result is in close agreement with differences in the structures determined by x-ray crystallography. We demonstrate additionally that when the same solutes that were used in competition experiments are used to probe changes accompanying the binding of free BamHI to its specific DNA sequence, the measured number of water molecules released in the binding process is strikingly solute-dependent (with up to 10-fold difference between solutes). This result is expected for reactions resulting in a large change in a surface exposed area.

Whereas it is generally accepted that hydration water plays an important role in DNA–protein sequence-specific recognition (1) there are only few techniques available to probe its contribution reliably. We use an approach termed the osmotic stress technique (2, 3) that measures changes in hydration coupled with changes in the functional state by measuring the effect of water activity on reaction equilibria and kinetics. The osmotic stress technique has been used previously to measure the changes in hydration accompanying the DNA binding of several regulatory proteins: Escherichia coli gal (4), lac (5), tyr (6), and λ Cro (7) repressors, E. coli CAP protein (8), Hin recombinase (9), Ultrathorax and Deformed homeodomains (10), the restriction endonucleases EcoRI (11–14), BamHI (15, 16), and EcoRV (17), HhaI methyltransferase (18), Sso7d protein (19), the TATA-binding protein (20, 21), and the E2C protein from papillomavirus (22).

The dependence of an equilibrium constant on the bulk solution osmotic pressure that is varied by adding neutral solutes that do not bind directly to the DNA or protein gives a difference in the number of associated waters between the initial reactants and the final products. More precisely, water is considered associated with the DNA, protein, and complex if it excludes osmolyte, otherwise there is no osmotic imbalance. Obviously, water that is sterically sequestered in cavities, channels, or pockets fulfills this requirement. All solutes that are excluded from these cavities act identically on the equilibrium. Water molecules hydrating exposed macromolecular surfaces are more problematic. For the most part, solutes are excluded from these waters due to “preferential hydration,” macromolecules prefer their interactions with water over osmolyte. Of course, the interaction energy of solute and surface will depend on the size and chemical nature of the osmolyte. The extent to which these macromolecular surfaces prefer water over osmolyte must therefore depend sensitively on solute size and nature, i.e. the number of waters associated with surface hydration depends on the osmolyte probing the surface. There are now many examples in literature establishing this sensitivity (2, 23–30). Some researchers emphasize the excluded solute rather than the included water, but the thermodynamics is the same and converting between the numbers of excluded solute and included water is straightforward (3).

Most applications of osmotic stress to DNA-binding proteins have examined changes in hydration linked to binding of free protein and DNA to form a specific complex. This reaction is necessarily connected to a large change in solution-exposed surface area and the change in hydration observed should be sensitive to the osmolyte used to set water activity. We have been particularly interested in differences in hydration between specific, noncognate, and nonspecific DNA-protein complexes rather than between free DNA and protein and complex. Fundamentally, we have been particularly interested in the link between sequestered water and binding free energies. Practically, relative binding constants are not only easier to measure but are also more directly relevant to binding specificity. The equilibrium for specific sequence DNA-binding proteins within the DNA-rich cellular environment of the cell or nucleus is most likely not between free and bound proteins, but between specifically and nonspecifically bound proteins. Finally, by directly comparing protein binding to specific and nonspecific DNA sequences we hope to avoid to a significant extent the contribution from changes in the exposed surface area that accompanies the binding of free protein.
We have focused particularly on the role of water in DNA sequence recognition of type II restriction endonucleases. This system is a paradigm for recognition stringency. The restriction endonuclease EcoRI binds to its canonical site, GAATTC, with a constant ~10^{11} M^{-1} in 100 mM salt but decreases by at least 1000-fold when any one of the 6 bp is changed (31). The crystal structure of the specific EcoRI-DNA complex shows practically anhydrous contact between the two surfaces (32, 33). We showed previously that a nonspecific complex of the EcoRI sequesters about 110 water molecules more than the complex with the specific recognition sequence (12). This difference in number of waters between specific and nonspecific complexes is only weakly sensitive to the solute identity, suggesting that these 110 waters are sequestered in a space that is sterically inaccessible to solutes, most likely at the EcoRI-DNA interface of the nonspecific complex. A structure for the nonspecific complex of the EcoRI is not available, precluding a definitive confirmation of the osmotic stress results. X-ray structures, however, for both specific, GGATCC, and noncognate, GAATTC, complexes of a closely related type II restriction endonuclease, BamHI, have been solved (34, 35). Unlike the extensive, direct protein-DNA contacts seen in the specific complex structure, the nonspecific complex structure shows a distinct gap between the protein and DNA major groove surfaces. The volume of the cavity seen at the interface of the nonspecific complex is ~4763 Å⁢³ in comparison to only ~282 Å³ at the interface of the specific complex. Only a very few water molecules are ordered enough in this region to be seen in the structure. Given a normal water density, ~30 Å³/molecule, the extra volume at the interface of the noncognate complex corresponds to about 150 water molecules.

Lynch and Sligar (15), however, concluded from the osmotic pressure dependence of the binding constant of free BamHI to the specific DNA sequence that only a few (~20) water molecules are released in the process of complex formation. Our goal here is 2-fold: 1) to measure directly differences in sequestered water between specific and nonspecific BamHI complexes to confirm the correspondence between waters measured by osmotic stress and structure; and 2) to demonstrate the complications connected with using osmotic stress for reactions that are accompanied by significant changes in exposed surface area as, for example, the specific binding of free protein. We measure the ratio of specific and nonspecific binding constants, $K_{nsp-sp}^2$, by direct competition assayed with a novel self-cleave assay developed by us (16). The osmotic pressure dependence of $K_{nsp-sp}^2$ indicates that about 120–150 extra water molecules are retained in the nonspecific versus specific BamHI-DNA complex in very good agreement with the x-ray structural data. This number is only slightly dependent on the nature of the solute used to set water activity for seven osmolytes, consistent with a steric exclusion of solutes from a cavity at the interface of the BamHI-DNA nonspecific complex. Small solutes such as methanol and glycerol show a smaller number of waters indicating they are able to penetrate the cavity.

We additionally demonstrate that when the same solutes are used to probe changes in hydration accompanying the binding of free BamHI to its recognition sequence, the measured difference in the number of participating water molecules is strikingly solute-dependent as expected for a reaction coupled with a significant change in exposed surface area. For example, the absolute specific binding constant of BamHI shows a weak dependence on osmotic pressure of the disaccharide sucrose translating into the release of only about 46 water molecules in forming the complex. In contrast, the tetrasaccharide stachyose has a very dramatic effect on specific binding, corresponding to the release of ~470 water molecules. This 10-fold difference in the numbers of waters associated with specific binding should be compared with differences in the number of waters of only ~126 and ~145 for sucrose and stachyose for the difference between specific and nonspecific complexes measured by competition.

EXPERIMENTAL PROCEDURES

Materials—A 349-bp DNA fragment containing a single BamHI-specific site was purified from PvuII digestion of a pNBE193 plasmid. Plasmid DNA and restriction enzyme PvuII were purchased from New England Biolabs.

The sequences of the double-stranded 30-bp long BamHI-specific sequence and nonspecific sequence competitor oligonucleotides used were: 5′-CCGGAGACTGCGAGTTAAGGGAGGTC-3′ and 5′-CCGGAGACTGCGCCTA-GGTTAAGGGAGT-3′, respectively. The BamHI specific recognition sequence is shown in bold letters. The BamHI nonspecific sequence oligonucleotide contains the inverted BamHI recognition sequence (bold, underlined) in place of the specific recognition sequence.

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

DNA binding and cleavage experiments were performed with highly purified BamHI restriction endonuclease (a kind gift of Dr. A. Aggarwal). Active protein concentrations were determined by direct titration with the 349-bp DNA fragment containing the corresponding specific recognition sequence under conditions of stoichiometric binding as described previously (12, 14).

Glycine betaine was purchased from U. S. Biochemical; α-methyl glucoside, stachyose, and trimethylamine N-oxide from Sigma; triethylene glycol from Fluka; sucrose and glycine from ICN Biomedicals; glycerol and methanol from Mallinck-
rodt; Me₂SO (dimethyl sulfoxide) from Calbiochem; and DL-threitol from Aldrich. All solutes were used without further purification. Osmolal concentrations of solutes were determined by direct measurement using a vapor pressure osmometer operating at room temperature (Wescor, Logan, UT, model 5520XR). Changes in water chemical potentials are linearly proportional to solute osmolal concentrations, i.e., \( \Delta \mu_w = \mu_w - \mu_w^{\text{ref}} = -RT [\text{osmolal}]/55.6 \), where \( \mu_w \) and \( \mu_w^{\text{ref}} \) are the water chemical potentials of the solutions with and without added osmolyte. Osmolal concentrations of highly volatile solutes such as methanol and Me₂SO were taken as equal to their molar concentrations. Glycine was also tried as an osmolyte but showed an effect on the dissociation kinetics of the BamHI-DNA specific complex that likely indicates direct binding. This was therefore not used for further experiments.

**Equilibrium Binding Experiments**—In DNA-BamHI binding experiments samples contained 20 mM imidazole (pH 7.0), 2 mM dithiothreitol, 50 \( \mu \)g/ml acetylated bovine serum albumin, and 0.1 mM EDTA. The salt concentration was varied between 60 and 220 mM KCl. The total reaction volume was 30 \( \mu \)l. Samples were incubated at 20 °C.

Specific-nonspecific equilibrium competition experiments were performed as described previously (12–14). Briefly, mixtures of BamHI (1.5 nM), the specific site fragment (3 nM), and the nonspecific oligonucleotide competitor (between 0 and 150 \( \mu \)M in oligonucleotide or 0–1.5 \( \mu \)M in bp), were incubated at 20 °C for time periods long enough to reach an equilibrium for the experimental conditions used. Control kinetic experiments were performed to determine half-life of the complex for different experimental conditions. The loss of specific site binding with increased nonspecific competitor DNA concentration was determined by either a self-cleavage assay or the gel mobility shift assay as described below.

**Dissociation Kinetics**—Solution conditions for BamHI kinetic experiments were 20 mM imidazole (pH 7.0), 2 mM dithiothreitol, 50 \( \mu \)g/ml bovine serum albumin, and 0.1 mM EDTA; the salt concentration range was 20 to 180 mM KCl. The experimental protocol followed a standard method for measuring dissociation kinetics. BamHI (1.5 nM) was initially incubated with the 349-bp DNA fragment containing the specific BamHI recognition site (3 nM) under conditions of virtually stoichiometric binding at 20 °C for 20 min. BamHI-specific sequence oligonucleotide was then added to a final 200-fold excess concentration of the recognition site and the reaction mixture incubated at 20 °C for different times. The fraction of specifically bound BamHI was assayed using a self-cleavage assay.

**Self-cleavage Assay**—The self-cleavage assay was performed as described in Sidorova et al. (16). We use the nuclease activity of restriction endonucleases to sensitively measure their specific binding. At sufficiently high concentrations of neutral osmolytes (such as glycine betaine, triethylene glycol, \( \alpha \)-methyl glucoside, etc.) to slow the complex dissociation rate and an excess of sequence-specific oligonucleotide to trap free enzyme, the cleavage reaction is triggered only at those DNA sites that already have bound enzyme. Under these conditions the fraction of specific DNA fragment cleaved reflects the fraction of DNA bound to the enzyme before the initiating nuclease reaction.

The cleavage mixture containing \( \text{MgCl}_2 \), glycine betaine, and specific sequence oligonucleotide was added to pre-equilibrated samples. The composition of the cleavage mixture was adjusted to ensure the presence of 10 mM \( \text{MgCl}_2 \) and 200-fold molar excess of the specific oligonucleotide competitor over the fragment after mixing. The salt concentration in the cleavage mixture was adjusted to ensure a final 60 or 100 mM KCl concentration. Enough glycine betaine was in the cleavage mixture to ensure a final osmotic pressure of at least 2 osmolal (for 60 mM KCl) or 3 osmolal (for 100 mM KCl) (16). Samples were incubated with cleavage mixture for 30 min at 20 °C; the cleavage reaction was then quenched by adding EDTA to a final concentration of 20 mM. In kinetic experiments, specific oligonucleotide was omitted from the cleavage mixture because a 200-fold excess of the specific oligonucleotide was already present in the reaction mixture. DNA digestion products were purified using GenElute PCR Clean-up kit (Sigma).

**Gel Mobility Shift Experiments**—To prevent re-equilibration of the samples in the electrophoretic well prior to entering the gel, a quench reaction protocol was used to “freeze” the equilibrium fraction of specifically bound protein (16). 30 \( \mu \)l of reaction quench mixture was added to each sample before loading on the gel. The composition of this quench mixture was such as to ensure that the final samples had a 200-fold molar excess of the specific site oligonucleotide compared with the specific site DNA fragment, 2 osmolal glycine betaine, and 2 mM Ca²⁺. The increased osmotic pressure and presence of Ca²⁺ lengthens the dissociation half-life of the BamHI-specific complex to several hours at least (16). In the absence of Mg²⁺ we observed no measurable cleavage of the DNA in the presence or absence of Ca²⁺.

**Gel Electrophoresis**—Ficoll was added to the DNA fragments from the self-cleavage assay to a final concentration of 3% and samples were loaded on a 10% polyacrylamide gel, TAE (22.5 mM Tris, 11.25 mM acetic acid, 0.5 mM EDTA, pH 8.3) buffer. Samples were run at 250 V for 3–4 h.

20 mM HEPES (pH 6.7) buffer mixed with 2 mM CaCl₂ was used in gel mobility shift experiments. CaCl₂ was present both in the gel and in the running buffer to stabilize the complex. BamHI samples were loaded on the gel at 230 V, and the gel was run for 3–4 h at this voltage.

The DNA in the gels was stained with the fluorescent dye SYBR Green I (Molecular Probes). The gels were imaged with a Luminescent Image Analyzer LAS-1000 plus (Fuji Film) that includes a 1.3 megapixel cooled CCD camera, epillumination at 470 nm (LED), and a dichroic optical filter suitable for SYBR Green I. The LAS-1000 plus was interfaced to a Pentium PC. Band intensities were quantified using Image Gauge (version 3.122) for Windows. The linearity of DNA fluorescent staining over the range of DNA concentrations studied was confirmed using pBR322 DNA fragments generated by MspI digestion.

**Equilibrium Competition and Kinetics Data Analysis**—As was developed previously (12), the ratio of specific (sp) and nonspecific (nsp) association binding constants \( (K_{sp}/K_{nsp}) \) can be determined from the loss of specifically bound complex as...
the concentration of a nonspecific oligonucleotide competitor is increased. If \( f_b \) and \( f_b^0 \) are the fractions of the protein-bound specific sequence fragment with and without added oligonucleotide competitor, respectively, then under conditions of virtually stoichiometric protein binding (<5% free protein) and for much weaker nonspecific than specific binding \( (K_{sp} \ll K_{sp}) \), the concentration of nonspecific complex can be easily calculated as equal to \( (f_b^0 - f_b)\cdot[\text{DNA}_{sp}]_{\text{total}} \). The specific sequence binding is given by Equation 1,

\[
f_b = f_b^0 - \frac{K_{sp}}{K_{sp}} f_b \frac{[\text{DNA}_{sp}]_{\text{total}}}{1 - f_b [\text{DNA}_{sp}]_{\text{total}}} \quad \text{(Eq. 1)}
\]

where \([\text{DNA}_{sp}]_{\text{total}}\) is the molar concentration of the specific sequence fragment, and \([\text{DNA}_{sp}]_{\text{total}}\) is the molar concentration of nonspecific oligonucleotide. Relative binding constants, \( K_{sp}/K_{sp} \), were straightforwardly calculated from the linear dependence of \( f_b \) on \( f_b:\text{DNA}_{sp}]_{\text{total}}/((1 - f_b)\cdot[\text{DNA}_{sp}]_{\text{total}}) \), measured at constant specific sequence DNA and protein concentrations.

Specific binding constants were determined for experimental conditions of nonstoichiometric protein binding, for example, in the presence of high salt, from Equation 2,

\[
K_{sp} = \frac{f_b}{1 - f_b} \cdot \frac{1}{[P]_{\text{free}}} = \frac{f_b}{(1 - f_b) \cdot (f_{b,\text{stoich}} - f_b)} \cdot \frac{1}{[\text{DNA}_{sp}]_{\text{total}}} \quad \text{(Eq. 2)}
\]

where \( f_{b,\text{stoich}} \) is the fraction of protein-bound DNA under conditions of stoichiometric binding. In 60 mM KCl, >95% of added protein is bound to the specific site DNA fragment.

We also performed equilibrium competition experiments under nonstoichiometric binding conditions (100 and 180 mM KCl) to overlap with specific binding experiments. Again \( f_b \) and \( f_b^0 \) are the fractions of DNA fragment with bound BamHI in the presence and absence of added oligonucleotide competitor, respectively. In this case, the concentration of nonspecifically bound protein, \([\text{DNA}_{sp} \cdot P]\), cannot be calculated directly from the loss of specific binding, \( (f_b^0 - f_b)\cdot[\text{DNA}_{sp}]_{\text{total}} \). The concentration of a free protein, \([P]_{\text{free}}\), however, can be directly calculated from the loss of specific binding because the specific association constant does not depend on competitor concentration. \( K_{sp} \) can be determined from binding in the absence of competitor (Equation 2) and used to determine the free protein concentration with added competitor,

\[
[P]_{\text{free}} = \frac{f_b}{K_{sp}(1 - f_b)} \quad \text{(Eq. 3)}
\]

The concentration of nonspecific complex and, consequently, the relative specific-nonspecific binding constant can then be straightforwardly calculated to,

\[
[\text{DNA}_{sp} \cdot P] = (f_{b,\text{stoich}} - f_b)[\text{DNA}_{sp}]_{\text{total}} - [P]_{\text{free}} \quad \text{(Eq. 4)}
\]

and,

\[
K_{sp-sp} = \frac{K_{sp}}{K_{sp}} = \left(\frac{f_b [\text{DNA}_{sp}]_{\text{total}}}{(1 - f_b) [\text{DNA}_{sp} \cdot P]} \quad \text{(Eq. 5)}
\]

where again \( f_{b,\text{stoich}} \) is the fraction of bound specific DNA under conditions of stoichiometric binding.

The difference in the numbers of solute excluding waters between specifically and nonspecifically bound protein can be calculated from the dependence of \( K_{sp}/K_{sp} \) on the solute osmolal concentration (2, 3) by,

\[
\frac{d \ln(K_{sp})}{d[\text{osmolal}]} = \frac{d \ln(K_{sp}/K_{sp})}{d[\text{osmolal}]} = \frac{\Delta N_{w,sp} - \Delta N_{w,nsp}}{55.6} \quad \text{(Eq. 6)}
\]

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

\[
\frac{d[\text{DNA}_b]}{dt} = -k_{\text{off}}[\text{DNA}_b]_p \quad \text{(Eq. 8)}
\]

where \([\text{DNA}_b]\) corresponds to the concentration of complex and \( k_{\text{off}} \) is the dissociation rate constant. The standard solution of this equation is,

\[
\ln \left(\frac{f_b}{f_b^0}\right) = -k_{\text{off}}t \quad \text{(Eq. 9)}
\]

where \( f_b \) is the fraction of specific complex at time \( t \) and \( f_b^0 \) is the fraction of complex at \( t = 0 \).

**RESULTS**

*The Osmotic Dependence of the Relative (\( K_{sp-sp} \)) BamHI Binding Constant*—To establish the times necessary to reach equilibrium, particularly for competition experiments, we performed control experiments analogous to those described in Ref. 16 to measure dissociation rates of the specific complex between BamHI and the 349-bp specific site DNA fragment (data not shown). In competition experiments, samples were equilibrated for a period of time at least 5-fold longer than the half-life time of the BamHI-DNA specific complex for each experimental condition. The BamHI dissociation rate from its specific sequence on DNA strongly decreases with increasing concentrations of neutral osmolytes (glycine betaine, \( \alpha \)-methyl glucoside, triethylene glycol, etc.) used to set bulk water activity (data not shown) and, as expected, strongly increases with increasing salt concentrations.

In the equilibrium competition experiments, mixtures of BamHI (~1.5 nM), the specific site fragment (~3 nM), and the
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FIGURE 1. Equilibrium competition for BamHI binding between specific and nonspecific DNA. Mixtures of BamHI, the 349-bp DNA fragment with a specific recognition site, and the nonspecific oligonucleotide competitor, were incubated in the presence of 0.1 or 0.7 osmolar α-methyl glucoside, 60 mM KCl, and 20 mM imidazole (pH 7.0) at 20 °C for at least 4 h. A, the loss of specific site binding as the concentration of competitor increased was determined by the self-cleavage assay. DNA fragments with initially bound enzyme are cleaved, but not free DNA fragments. Binding is assayed through the fraction of DNA fragment that was cleaved. Fig. 1A shows a gel image illustrating the competition for BamHI binding between a nonspecific oligonucleotide and the specific BamHI sequence containing fragment for two concentrations of methyl glucoside. The fraction of cleaved DNA decreases with increasing concentration of the nonspecific competitor. Fig. 1B quantifies the competition. Under the experimental conditions used (60 mM KCl, 20 mM imidazole, pH 7.0) BamHI binds the specific fragment stoichiometrically allowing easy calculation of the relative binding constant (K_{nsp-sp} = K_{sp}/K_{nsp}) using Equation 1 under “Experimental Procedures.” The slope of the linear fit to the data gives the ratio of specific and nonspecific DNA binding constants. The dependence of the binding free energy difference between specific and nonspecific binding, ln(K_{nsp-sp}) = −ΔG/RT, where RT (∼0.6 eV/mol at 20 °C) is the thermal energy, on α-methyl glucoside osmotic concentration is shown in Fig. 1C. The dependence is linear with a slope equal to 2.4 (±0.2) that translates into ∼135 more water molecules retained in the nonspecific BamHI-DNA complex compared with the specific sequence complex (see Equation 6). The linearity indicates that the difference in the number of waters between the specific and nonspecific complexes is independent of osmolyte concentration.

Competition experiments analogous to that illustrated in Fig. 1 were performed for six other solutes. The dependence of the binding free energy difference between specific and nonspecific BamHI binding on osmolyte concentration for the seven different solutes is shown in Fig. 2. Relative binding constants measured with a more traditional gel mobility shift assay using a quench reaction protocol as described under “Experimental Procedures” and in Ref. 16 have the same values as those measured with the self-cleavage assay.

Osmolyte concentrations varied between 0 and 0.8 osmolar for all solutes except stachyose. For stachyose we could perform measurements only up to 0.3 osmolar because the dissociation rate becomes too slow at higher concentrations of this solute. Changes in competitive binding free energies scale linearly with changes in osmolality (water activity) for each solute. The observed linearity is an important feature that allows distinguishing an indirect osmotic effect of the solute from direct solute binding with DNA or protein. The slopes of the lines can be easily translated into differences in the amount of water nonspecific oligonucleotide competitor (between 0 and ∼50 μM in oligonucleotide or ∼1.5 mM in bp), were incubated at 20 °C for times long enough to ensure equilibrium under the experimental conditions used. The loss of specific site binding as the concentration of nonspecific competitor DNA increased was determined by the self-cleavage assay. With this technique DNA fragments with initially bound enzyme are cleaved, but not free DNA fragments. Binding is assayed through the fraction of DNA fragment that was cleaved. Fig. 1A shows a gel image illustrating the competition for BamHI binding between a nonspecific oligonucleotide and the specific BamHI sequence containing fragment for two concentrations of methyl glucoside. The fraction of cleaved DNA decreases with increasing concentration of the nonspecific competitor. Fig. 1B quantifies the competition. Under the experimental conditions used (60 mM KCl, 20 mM imidazole, pH 7.0) BamHI binds the specific fragment stoichiometrically allowing easy calculation of the relative binding constant (K_{nsp-sp} = K_{sp}/K_{nsp}) using Equation 1 under “Experimental Procedures.” The slope of the linear fit to the data gives the ratio of specific and nonspecific DNA binding constants. The dependence of the binding free energy difference between specific and nonspecific binding, ln(K_{nsp-sp}) = −ΔG/RT, where RT (∼0.6 eV/mol at 20 °C) is the thermal energy, on α-methyl glucoside osmotic concentration is shown in Fig. 1C. The dependence is linear with a slope equal to 2.4 (±0.2) that translates into ∼135 more water molecules retained in the nonspecific BamHI-DNA complex compared with the specific sequence complex (see Equation 6). The linearity indicates that the difference in the number of waters between the specific and nonspecific complexes is independent of osmolyte concentration.

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The salt dependence of the BamHI specific binding. Complexes of the specific site DNA fragment and BamHI were prepared at different KCl concentrations in the absence of osmolytes. A, the fraction of DNA with bound BamHI at each salt concentration was measured using the self-cleavage assay. Protein binding is essentially stoichiometric for salt concentrations less than about 90 mM KCl. The fractions of BamHI-bound DNA measured at 60 and 80 mM KCl were used therefore to determine the total amount of active protein present in the reaction mixture. B, the dependence of the specific association binding constant on the KCl concentration is shown. This dependence is linear over the whole range of salt concentrations used (100–240 mM KCl) and corresponds to a release of 5.3 (±0.6) K⁺ ions coupled to specific BamHI binding.

The salt dependence of BamHI-DNA equilibrium binding was measured for the range of salt concentrations from 60 to 240 mM KCl. Protein was added to specific DNA fragments, and the complex incubated at 20 °C for periods of time long enough to reach equilibrium. No osmolytes were added to the reaction mixture. The salt titration curve is shown in Fig. 3A. With increasing salt concentration the fraction of bound DNA decreases. At 180 mM KCl only ~15–20% of added protein is bound to DNA. BamHI binding is virtually stoichiometric up to ~90 mM KCl, so fractions of the cleaved DNA fragments measured at 60 and 80 mM KCl were used to estimate the total amount of active protein in the reaction mixture. The specific binding constant of BamHI-DNA binding can then be easily calculated for each salt concentration (Equation 2). The salt...
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FIGURE 4. Neither the difference in binding free energy nor the difference in the number of sequestered water molecules between specific and nonspecific complexes is sensitive to salt concentrations between 60 and 180 mM KCl. Relative binding constants, $K_{	ext{nsp-sp}},$ were determined at 60 (○), 100 (□), and 180 mM (●) KCl as dependent on the osmotic concentration of glycine betaine. The relative binding constants in the absence of applied osmotic stress, $K_{	ext{nsp-sp}}^\text{osm}$, and the difference in the number of sequestered water molecules, $\Delta N_{\text{w,sp}}$, calculated from the slopes and intercepts of best fitting lines to the data differ less than 3%.

dependence of the specific BamHI-DNA binding constant in the 100 to 240 mM KCl range is shown on Fig. 3B. Dependence of $\log(K_{\text{sp}})$ on $\log([\text{KCl}])$ is linear over this range of KCl concentrations with a slope approximately $-5.3$. This number is only slightly different from the $-4.4$ slope of $\log(K_a)$ versus $\log([\text{KAc}])$ dependence reported by Engler et al. (36).

We also measured the salt and osmotic pressure dependence of nonspecific-specific BamHI binding competition for three salt concentrations, 60, 100, and 180 mM KCl (Fig. 4), and with glycine betaine. In contrast to the specific binding constant, the relative nonspecific-specific binding constant has a very weak salt sensitivity. Engler et al. (36) report that nonspecific binding of BamHI is more sensitive to ionic strength than its specific binding with about 6.1 K$^+$ ions released in the process of nonspecific complex formation or $\sim1.7$ ions more than specific binding.

The osmotic dependence of $K_{\text{nsp-sp}}$ measured at these three salt concentrations is practically indistinguishable between 0 and 1 osmolal glycine betaine. The slopes of the linear fits of $\ln(K_{\text{nsp-sp}})$ versus betaine osmolal concentration are the same for each salt concentration within experimental error and equal to 2.4 ($\pm0.2$).

Finally, we measured the salt dependence of the BamHI dissociation rate constant from its specific sequence in KCl concentrations ranging from 20 to 100 mM. This experiment is illustrated in Fig. 5. The log-log plots of $k_{\text{off}}$ versus salt concentration is linear with the slope corresponding to $\sim4.5$ K$^+$ ions thermodynamically coupled to dissociation. No deviation from linearity was observed over the entire salt concentration range examined. Dissociation rates are too fast to measure by our method at KCl concentrations above $\sim60$ mM in the absence of osmolyte (half-life time of the BamHI-DNA complex is $\sim4.4$ min in 60 mM KCl, pH 7.0). The dissociation rate measured at 100 mM KCl shown in Fig. 5 was obtained with added glycine betaine and the rate extrapolated to 0 osmolal (data not shown).

The Osmotic Dependence of the BamHI-specific Binding Constant Is Solute-dependent—The effect of different osmolytes on BamHI-specific binding was measured at 180 mM KCl. The total amount of active protein present in the mixture was determined in parallel control experiments from specific site binding at 60 mM KCl, i.e. under conditions of stoichiometric BamHI-DNA binding. Specific binding constants measured without added osmolyte were insensitive (to within 3%) to the ratio of BamHI and DNA concentrations over a 4-fold range. Fig. 6A shows a gel image illustrating the increase in specific binding (increase in fragment cleavage) as the concentration of triethylene glycol increases for 180 mM KCl (lanes 1–7). Fig. 6B shows the dependence of the fraction of DNA fragment with bound enzyme, $f_p$, on triethylene glycol osmolal concentration for both 60 and 180 mM KCl. The dependence of the specific binding free energy on osmotic pressure is shown in Fig. 6C. The slope translates into $\sim183$ water molecules released in the process of complex formation if ethylene glycol is used to set water activity (compared with $\sim145$ water molecules measured in competition experiments using triethylene glycol).

The same experiment was then repeated with other solutes. Fig. 7 shows the osmotic dependence of the BamHI-specific binding constant for three sugars. Values for the specific binding constant measured without added solute for these three independent sets of experiments are within 10% error: $2.2 \times 10^8$ M$^{-1}$, $2.5 \times 10^8$ M$^{-1}$, and $2.4 \times 10^8$ M$^{-1}$. The osmotic dependence of the specific binding constant is linear for all solutes again indicating an osmotic action rather than direct solute binding to DNA or protein. The numbers of water molecules, $\Delta N_{\text{w,sp}}$, released in complex formation are now strikingly different ranging from $\sim46$ molecules using sucrose to set water...
activity to ~470 water molecules seen with stachyose. In contrast, the inset to Fig. 7 shows that \( \text{Nw,nsp-sp} \) is virtually insensitive to the identity of the solute for the same three sugars.

Table 1 summarizes the osmotic stress results for both specific and specific-nonspecific competitive binding for six osmolytes. The weak (10% at the most) sensitivity of \( \text{Nw,nsp-sp} \) on solute identity is in contrast with quite a disperse number of waters released with binding of free protein to its specific sequence.

There Is a Size Limit for Solute Exclusion from the Interfacial Cavity—The seven solutes (\(-\text{methyl glucoside, sucrose, stachyose, glycine betaine, Me}_2\text{SO, trimethylamine N-oxide, and triethylene glycol}\)) used in Fig. 2 showed very similar differences in the number of waters retained by the nonspecific compared with the specific BamHI-DNA complex, 133 (±3) waters. This slight dependence on size and nature of the solute is characteristic of steric exclusion. Indeed, changing the sugar size from the monosaccharide \(-\text{methyl glucoside (Mr 194)}\) to the disaccharide sucrose (\(\text{Mr 342}\)) to the tetrasaccharide stachyose (\(\text{Mr 667}\)) does not observably change the number of excluded waters probed in competition experiments (Figs. 2

**FIGURE 6.** Specific binding of BamHI is strongly promoted by triethylene glycol. A, a gel image is shown illustrating the increased stability of the BamHI-DNA specific complex with increasing triethylene glycol concentrations. In lanes 1–7, the self-cleavage assay was used to quantify BamHI binding at 180 mM KCl with varied triethylene glycol osmotic pressure. Increased specific binding is apparent from the increasing fractions of DNA fragments cleaved. In lanes 8–10, BamHI binding under stoichiometric conditions, 60 mM KCl, was monitored with the self-cleavage assay at three triethylene glycol osmolar concentrations. B, the fraction of cleaved (bound) DNA is shown plotted against the triethylene glycol osmolar concentration for samples prepared in 180 mM KCl (○) and for control samples prepared in 60 mM KCl (△). Binding in the presence of 180 mM KCl becomes virtually stoichiometric for triethylene glycol concentrations higher than ~1.4 osmolar. BamHI binding is stoichiometric at 60 mM KCl even in the absence of triethylene glycol. The average fraction of bound DNA at 60 mM KCl was used to estimate the total amount of active protein present in the experimental mixture. C, the specific site binding free energy (\(\ln(K_{sp}) = -\Delta G_{sp}/RT\)) at 180 mM KCl is plotted versus triethylene glycol concentrations. The linear slope corresponds to the release of ~180 water molecules to the bulk solution coupled to the specific complex formation.
pockets, grooves, or cavities are often sterically inaccessible to solutes. In this most straightforward case, the chemical nature or the size of a solute after a certain size limit does not matter. There is simply osmotic pressure acting on a sequestered volume of water. The open-close reaction of membrane-incorporated channels is conceptually the simplest case to visualize the steric exclusion of the solutes from water-filled cavities (37, 38). Another example, however, is the specific-nonspecific reaction of DNA-binding proteins. We showed previously that about 110 extra water molecules (a number that only slightly depended on solute nature used to set water activity) are sequestered in the nonspecific compared with the specific complex of the restriction endonuclease EcoRI (12, 13). This number would correspond to a bit more than a full hydration layer of water at the interface of the nonspecific complex. The difference between specific and nonspecific complexes of BamHI is now another case as illustrated in Fig. 2. Seven solutes differing in size and chemical nature show practically indistinguishable osmotic effects (10–15% difference at the most) on specific-nonspecific competition binding of BamHI-DNA. The best fit to all the data gives $\Delta N_{\text{w,sp-nsp}} \approx -130(\pm 7)$ water molecules. This number is in excellent agreement with the difference in structure between specific and nonspecific complexes determined by x-ray crystallography (34, 35). The difference in the cavity volumes at the BamHI-DNA interface corresponds to about 150 water molecules.

Just as with membrane channels (37, 38), however, solutes that are small enough can penetrate even very tight spaces as illustrated in Fig. 8. Methanol, the smallest solute examined, has the weakest effect on the relative BamHI binding constant. Methanol is excluded by a difference of only 21 (±5) water molecules between the nonspecific and specific complexes. Glycerol is larger and correspondingly more excluded, showing a difference of 64 (±7) waters between the nonspecific and specific complexes. The difference with threitol ($M_r = 122$) is 95 (±9) water molecules. The larger polyols, α-methyl glucoside, the disaccharide sucrose, and tetrasaccharide stachyose give 135 (±11), 126 (±8), and 145 (±13) water molecules, respectively, retained in the nonspecific versus specific BamHI-DNA complex. The plateau of about 130 waters now remains almost the same not only for these polyols, but also for solutes of different chemical natures (Fig. 2).

Water molecules that hydrate exposed protein and DNA surfaces, however, exclude solutes differently from sterically sequestered water. The number of waters probed by the solute depends on competition between water molecules and solute for interaction with macromolecules. Consequently, this number depends both on solute size and its chemical nature. There are two mechanisms commonly considered. Crowding (39, 40) recognizes that there is steric exclusion of large solutes from surfaces; solutes cannot approach as closely as water molecules, this effect of solute exclusion increases with solute size. Preferential hydration (24, 41) recognizes that the interaction of water with groups on nucleic acid and protein surfaces may be more energetically favorable than interaction of solutes with macromolecules. Many experiments measuring both exclusion of solutes from protein and DNA surfaces as well as the effect of this exclusion on different macromolecular reactions show that the

### Differences in Hydration Coupled to BamHI-DNA Binding

#### TABLE 1
The osmotic stress results for both specific and specific-nonspecific competitive BamHI-DNA binding are shown for six solutes

| Solute                | $\Delta N_{\text{w,sp-nsp}}$ | $\Delta N_{\text{w,sp}}$ |
|-----------------------|------------------------------|---------------------------|
| Methyl glucoside      | 135 ± 11                     | 109 ± 10                  |
| Sucrose               | 126 ± 8                      | 46 ± 10                   |
| Stachyose             | 145 ± 13                     | 467 ± 33                  |
| Betaine               | 132 ± 10                     | 135 ± 25                  |
| Me$_2$SO              | 120 ± 5                      | 158 ± 8                   |
| Triethylene glycol    | 145 ± 22                     | 183 ± 9                   |

#### FIGURE 8. Small solutes are able to partially penetrate the nonspecific complex interfacial cavity. The relative specific-nonspecific binding free energy is plotted against the osmolar concentration for several alcohols and polyols: stachyose (●), 145 (±12) waters; α-methyl glucoside (○), 135 (±11) waters; sucrose (□), 126 (±8) waters; threitol (●), 95 (±9) waters; glycerol (□), 64 (±7) water molecules; and methanol (△), 21 (±5) water molecules. Conditions were: 60 mM KCl and 20 mM imidazole (pH 7.0) for all solutes except stachyose. Measurements for stachyose were performed at 100 mM KCl.

and 7, inset). Of course, there must be some size small enough that solutes can begin to penetrate the cavity. Fig. 8 demonstrates that indeed when threitol ($C_4H_{10}O_4$, $M_r = 122$) is used to set water activity the difference between nonspecific and specific complexes is measured as only about ~95 waters instead of ~130 water molecules; glycerol ($C_3H_8O_3$, $M_r = 92$) shows ~64 waters. Methanol ($CH_3OH$, $M_r = 32$), the smallest alcohol examined in this series, has an even weaker effect on the competitive binding constant. The slope of $\ln(K_{\text{nsp-sp}})$ versus methanol osmolar concentration corresponds to only a difference of ~21 water molecules between the specific and nonspecific complexes. The simplest explanation would be that threitol, glycerol, and methanol are able to penetrate the interface cavity of the nonspecific complex and are therefore only partially excluded from these waters.

### DISCUSSION

There are generally two classes of water that must be considered in interpreting the effect of osmotic stress on DNA-protein binding reactions. The change in hydration that occurs in the course of the binding reaction is the change in the number of water molecules that are unable to dissolve a particular solute or, equivalently, that exclude solute. Water sequestered in
apparent number or change in the number of hydrating waters is constant over a wide range of solute concentrations for each osmolyte, but that this number is sensitively dependent on the particular solute probing surface hydration (2, 25–30, 41). For the osmolytes we usually use (sucrose, glycine betaine, triethyleneglycol, etc.) a 2–5-fold difference in exclusion is not uncommon. Previously, Garner and Rau (4) measured the release of water correlated with binding of the free gal repressor from the bulk solution to its operator sequences. The osmotic sensitivity of the operator binding to repressor from solution showed a significant dependence on the chemical nature of the solute. The number of waters released in the process of binding of the Gal repressor to DNA varied from ~100 for betaine glycine to 180 for triethylene glycol. The osmotic sensitivity of the dissociation rate of the λ Cro repressor from different operator sequences (7) presents an even more dramatic example of solute dependence. There is a 6-fold difference in the number of waters coupled to the dissociation rate of the λ Cro protein from the weakest operator sequence examined between glycine betaine and triethylene glycol.

Unlike the specific-nonspecific binding competition, the specific binding of free BamHI to its DNA recognition sequence is accompanied by a large change in exposed surface area. The sensitivity of $\Delta N_{w,sp}$ on solute identity seen here confirms this. Fig. 7 shows specific equilibrium association constant dependence on osmotic pressure for α-methyl glucoside, sucrose, and stachyose. For each solute, the dependence is linear confirming an osmotic action of these solutes, but the number of water molecules released in the course of binding ranges from ~46 waters for sucrose up to ~470 waters for stachyose. This dramatic dependence of $\Delta N_{w,sp}$ for specific BamHI binding on saccharide identity stands in striking contrast to the solute insensitivity of $\Delta N_{w,sp}$ for specific-nonspecific binding equilibrium (Fig. 7, inset); further emphasizing the difference in osmolyte exclusion between sterically sequestered water and surface hydration. In fact, the small spread in values of $\Delta N_{w,sp}$ may reflect the slight difference in exposed surface seen in the x-ray structures between the nonspecific and specific complexes of BamHI in addition to the interfacial cavity.

Interestingly, the osmotic effect of saccharides on specific BamHI binding does not scale with solute size; the monosaccharide α-methyl glucoside shows stronger exclusion than the disaccharide sucrose. We previously observed that the change in water associated with the dissociation rate of nonspecifically bound EcoRI also strongly depended on solute nature (13). This reaction step is also likely characterized by a change in solute-accessible surface area. For α-methyl glucoside and stachyose an uptake of ~15 waters was coupled with the nonspecific EcoRI dissociation. With sucrose, in contrast, a release of ~10 water molecules was observed suggesting a small preferential inclusion of sucrose with the newly exposed surface area.

CONCLUSIONS

The osmotic stress technique can be used to reliably probe differences in the number of waters in sterically sequestered cavities at the interface of specific, nonspecific, and noncognate DNA-protein complexes. There are certain precautions that should be taken to ensure structurally meaningful results. A variety of solutes of different sizes and chemical natures should be used to probe differences in hydration. If several solutes give the same $\Delta N_{w}$ within 15–20%, then this number of waters can be taken as a meaningful measurement of the sterically sequestered interfacial cavity. As Fig. 8 shows quite convincingly, the use of solutes, as methanol and glycerol that are small enough to penetrate even sterically sequestered cavities, can result in an underestimation of the real number of sequestered waters.

Interpreting osmotic stress results for reactions that are characterized by large changes in solute accessible surface area as specific BamHI-DNA binding is an even more complicated task. Solute exclusion is sensitively dependent on osmolyte identity, as is apparent in the 10-fold difference in the numbers of released water molecules for specific complex formation using different solutes (Fig. 7 and Table 1). Clearly, it would be wrong to claim that absolute binding of BamHI to its specific sequence leads to release of 40 (or 100 or 400) water molecules unless it is stated clearly that this number was obtained with a particular solute. To obtain structural information from these solute-specific effects, the extent of exclusion of the solute from the macromolecule must be measured separately (42, 43). Exclusion is dependent not only on the solute size and nature, however, but also on the nature of the particular macromolecular surface. This might prove problematic for heterogeneous surfaces such as proteins.

The osmotic stress technique measures how many water molecules that exclude solute are released to the bulk solution coupled to a reaction. These numbers are quite real and important practically. If one, for example, would like to increase the stability of the specific BamHI-DNA complex, triethylene glycol or glycine betaine would obviously be a much better choice than sucrose. Such information can be very valuable in different experimental approaches and should not be dismissed because of its complexity.

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