FOR THE RECORD

Participation of water in Hin recombinase–DNA recognition

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Abstract: The participation of water molecules in the interaction between the Hin recombinase and its operator DNA has been detected by analysis of the dissociation constant in the presence of varying concentrations of neutral solutes and cosolvents. The dissociation constant as measured by gel mobility shift assays increased as the concentration of dimethyl sulfoxide, glycerol, sucrose, or polyethylene glycol was increased. Osmotic pressure is the only property that correlates with the change in the dissociation constant for all compounds. This data indicates that binding of a small population of water molecules accompanies formation of the Hin–DNA complex, and points to a novel role for solvent molecules in assisting site specific interaction between DNA-binding proteins and their cognate DNA sequence.

Keywords: osmotic pressure; solvation; protein–DNA interactions

Protein–DNA interactions are crucial in regulation of cellular function, both in the maintenance of normal growth and development and in the onset of disease. Elucidation of the origin of specificity in these macromolecular recognition events is crucial to understanding this class of interactions and in the generation of therapeutic agents and novel research tools.

Many investigators have been studying the role of water in mediating recognition in protein–DNA and protein–protein complexes. Water has been detected in several such systems, including the trp repressor–operator complex, *Eco*R I and other endonucleases, cytochrome P450, hemoglobin, and hexokinase. In some instances, a small population of water molecules is present in a cavity or interstitial space in a macromolecular complex (Otwinowski et al., 1988; Westhoff, 1988; Carey et al., 1991; DiPrimo et al., 1992; Bhat, 1993; Dzingeleski & Wolfenden, 1993; Lawson

& Carey, 1993; Qian et al., 1993; Feng et al., 1994; Zhang & Matthews, 1994). In other cases, evidence shows that large changes in solvation of macromolecular surfaces accompany or drive conformational changes or binding and recognition events (Kornblatt & Hui Bon Hoa, 1990; Colombo et al., 1992; Kuhn et al., 1992; Bezrukov & Vodyanoy, 1993; Rand et al., 1993; Oliveira et al., 1994; Robinson & Sligar, 1994, 1995a; Garner & Rau, 1995).

Recently, we and others have developed techniques of using osmotic stress to detect participation of water in molecular recognition (for reviews see Parsegian et al., 1995; Robinson & Sligar, 1995b). For a population of water molecules that are inaccessible to a neutral solute or cosolvent present in the bulk solvent (e.g., those located in a cavity, core, or interface, or associated with a protein surface), the chemical potential is controlled by the solution osmotic pressure. When the osmotic pressure is raised, equilibration of the chemical potential is established by the release of such water molecules into the bulk solvent (Tombs & Peacocke, 1974). If this change in solvation is coupled to a molecular transition such as binding, folding, or a conformation change, the equilibrium constant will shift with osmotic pressure, according to the principle of LeChatlier. Thus, a correlation between osmotic pressure and a macromolecular process is strong evidence for participation of water in at least one of the states (Rand, 1992; Parsegian et al., 1995). For example, release of water accompanies glucose binding by hexokinase and, as a result, increases in osmotic pressure yield tighter binding (Rand et al., 1993).

Using this approach, we have shown previously that waters play a significant role in accurate protein–DNA recognition in several restriction enzyme–DNA complexes (Robinson & Sligar, 1994, 1995a). Structural studies have also demonstrated that waters mediate contacts between repressor and operator DNA (Otwinowski et al., 1988; Lawson & Carey, 1993). Key questions of central importance to molecular biology are whether these features occur in other classes of DNA-binding proteins and enzymes, and whether interstitial waters detected in X-ray crystal structures always indicate water-mediated recognition. To address these questions, we have studied a system in which structural waters at the protein–DNA interface have been identified by crystallographic analysis, the Hin recombinase.

The Hin recombinase from *Salmonella typhimurium* is a member of the class of DNA-cleaving enzymes termed invertases. Hin recombinase is a 190-amino acid protein that catalyzes the inversion of a 1-kb segment of DNA in the *Salmonella* chromosome. This inversion controls the expression of two flagellin genes, leading to the regulation of flagellar phase variation (Hughes et al., 1992). The 52-amino acid DNA-binding domain of Hin binds to a 26-base pair site, making extensive contacts in the major and minor grooves of DNA (Bruist et al., 1987; Feng et al., 1994). A high-resolution X-ray crystal structure of this DNA-binding domain bound to a 14-base pair DNA fragment containing the cognate *hixL* site was reported recently (Feng et al., 1994). The structure has elements that are similar to both helix-turn-helix domains, and to eukaryotic homeodomain proteins. In the structure, two bound waters have been identified in the protein–DNA interface. These waters are involved in a network of hydrogen bonding interactions between the protein and DNA. The structure determination complimented exhaustive studies that measured binding affinity for all possible single site variants in the DNA binding sequence (Hughes et al., 1992). Based on these results, an explanation was proposed for the mechanism by which the interstitial waters assist recognition between the recombinase and its target DNA sequence (Feng et al., 1994). The observed network of hydrogen bonds made by the two waters to bases G9, A10, and T11 (numbering scheme according to Feng et al., 1994) is also possible for sequences GAA or GGG, but not for sequences GXC. Accordingly, Hin binds poorly to sequences containing a C at position 11.

In addition to Hin, the family of bacterial DNA invertases includes Gin from phage Mu, Cin from phage P1, and Pin from the *e14* prophage of *Escherichia coli*. Members of this family share substantial sequence identity and can complement one another functionally (Heichman & Johnson, 1990). The wealth of information accessible in these four systems make this evolutionary family an important model for protein–DNA interactions, particularly because the structure of the Hin–DNA complex is replete with examples of major groove, minor groove, and water-mediated interactions. Therefore, it is of interest to develop a precise understanding of the forces and molecular mechanisms for each feature of the recognition.

To test one aspect of the model for the origin of specificity in the Hin–DNA complex, we sought to determine whether water participates in recognition of cognate DNA by the Hin recombinase, using osmotic stress to perturb bound waters in the complex. In this study, we measured the strength of the binding between the DNA-binding region of the Hin recombinase (HinL) and the DNA binding site (*hixL*), as a function of solution osmotic pressure. We find that increases in osmotic pressure lead to decreases in the affinity of the HinL peptide for the *hixL* half-site. This study represents the first instance that we are aware of in which osmotic stress has been used in conjunction with gel shift analysis to detect water-binding associated with protein–DNA complex formation. These findings also represent the first solution evidence for water-mediated binding by an invertase and have general implications for the study of macromolecular interactions.

Results and discussion: To assay the effects of osmotic pressure on binding of the Hin peptide to cognate DNA, gel mobility shift assays were performed (Carey, 1991). It should be noted that several nonequilibrium factors can influence the outcome of gel-shift experiments. In particular, depending on the dissociation rate con-

stant for the complex relative to the rate at which the samples enter the gel matrix, mixing of the sample with running buffer could perturb the binding equilibrium away from its solution value. We have attempted to minimize this possibility by verifying that the measured value of the dissociation constant was not affected by the order in which the samples were loaded, or whether the samples were loaded with the current on or off. It has been shown previously that, even if the running buffer and sample buffers are of different composition, solution conditions within the sample determine the relative populations of free and bound DNA (Fried, 1989). Thus, the effects that osmotic pressure induce on the binding equilibrium in the samples are reflected in the outcome of the gel shift experiment.

Although it is of some concern that osmotic pressure itself might produce artifacts in the gel shift experiment, previous studies with other systems have shown a decrease in dissociations constant (K_d) as osmotic pressure is increased (Garner & Rau, 1995; Robinson & Sligar, 1995b; Sidorova & Rau, 1995; 1996). Because we observe an *increase* in K_d with increased osmotic pressure, it seems unlikely that a qualitative systematic error is uniformly associated with this approach.

To facilitate loading the gels, glycerol was present in the standard buffer, which induces an osmotic pressure of 5 atm. The values of 50 atm and 100 atm indicated are relative to the osmotic pressure in this standard buffer. The measured K_d values were independent of whether glycerol was incubated with the peptide and DNA or added just prior to loading.

Several features of the experiments indicate that the binding parameters measured in these experiments are for specific binding of cognate DNA by Hin peptide. The Hin peptide is capable of tight binding to its cognate DNA sequence even in the presence of excess nonspecific DNA. The peptide induces an observable shift in the electrophoretic mobility of *hixL* DNA (Fig. 1). The shifted bands are clear and sharp, characteristic of specific binding. Because the Hin peptide is capable of binding to a 13-base pair DNA fragment (Feng et al., 1994), the 26-base pair *hixL* fragment is expected to contain 14 essentially equivalent nonspecific sites. Therefore, nonspecific binding often results in fuzzy or indistinct bands and a loss of total intensity (Brown & Sauer, 1993). Under the conditions of the K_d determinations, bands corresponding to bound DNA were sharp and clear, with no loss of intensity. Nonspecific binding, evidenced by a loss of intensity of the shifted DNA band, or "supershifting" of the DNA to even slower mobility than the bound form, was only observed at protein concentrations in excess of 10^{-6} M, well above the concentrations used in determination of K_d values. No change in the extent of this nonspecific binding was detectable with the addition of solutes or cosolvents (data not shown).

In each gel, equivalent total density was observed in each lane. Moreover, the addition of cosolvents and solutes designed to increase osmotic pressure did not perturb the binding assay. The density observed in each lane at 0 atm, 50 atm, and 100 atm osmotic pressure were essentially equal. No density was observed in the wells, indicating that the presence of the osmolytes did not inhibit the ability of the free DNA or protein–DNA complex to enter the gel. Increased osmotic pressure resulted in an increase in the protein concentration needed for half-maximal binding (Fig. 2).

Binding isotherms were produced by analysis of the fraction of bound and free DNA in each gel shift experiment. The data fit well to Equation 1, indicating that binding is well-described by the

A

B

C
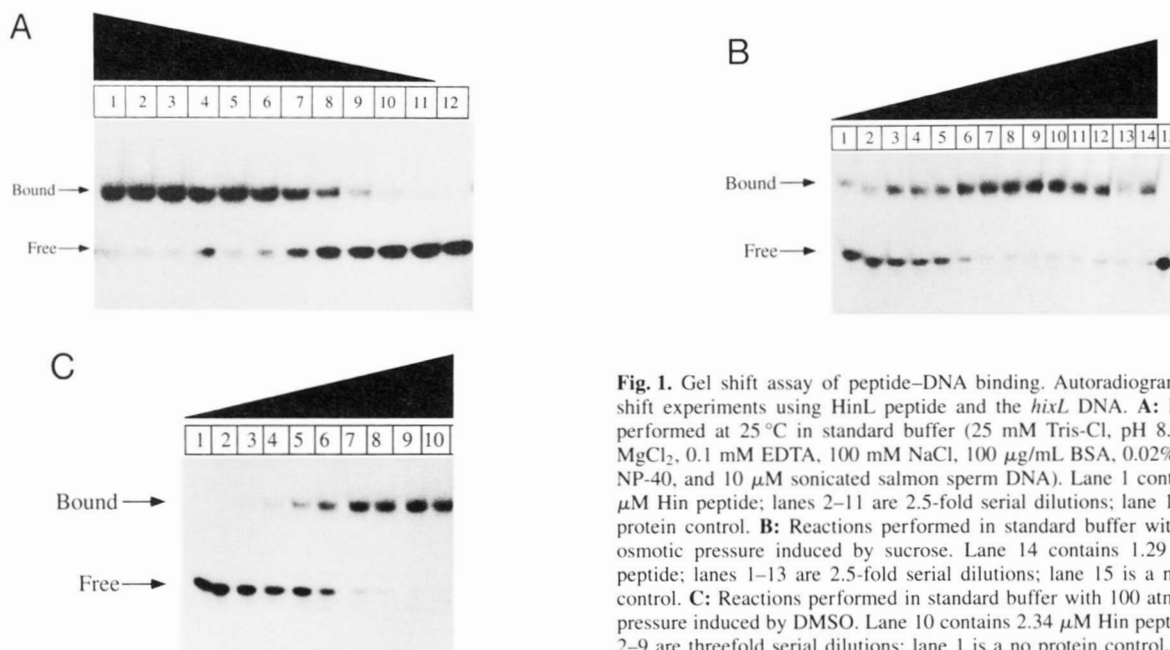
Fig. 1. Gel shift assay of peptide–DNA binding. Autoradiograms of gel shift experiments using HinL peptide and the *hixL* DNA. **A:** Reactions performed at 25 °C in standard buffer (25 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl, 100 μg/mL BSA, 0.02% Nonidet NP-40, and 10 μM sonicated salmon sperm DNA). Lane 1 contains 2.45 μM Hin peptide; lanes 2–11 are 2.5-fold serial dilutions; lane 12 is a no protein control. **B:** Reactions performed in standard buffer with 50 atm osmotic pressure induced by sucrose. Lane 14 contains 1.29 μM Hin peptide; lanes 1–13 are 2.5-fold serial dilutions; lane 15 is a no protein control. **C:** Reactions performed in standard buffer with 100 atm osmotic pressure induced by DMSO. Lane 10 contains 2.34 μM Hin peptide; lanes 2–9 are threefold serial dilutions; lane 1 is a no protein control.

equilibrium expression in Equation 2 (Fig. 2). At each osmotic pressure, and for each compound, binding data averaged from at least five independent measurements were fit to Equation 1 to determine the values of K_d, the equilibrium dissociation constant (Table 1).

We observe a significant decrease in binding affinity with the addition of neutral solutes and cosolvents. The K_d determined at 0 atm was approximately 10 nM, close to the value obtained in

previous studies (Hughes et al., 1992). The average of K_ds determined for each compound at 50 atm osmotic pressure was 16 ± 1 nM. At 100 atm, the average K_d was 22.2 ± 0.7 nM. Combining the data for all compounds and fitting as a single aggregate data set gave equivalent results within error. The decrease in affinity is well correlated with osmotic pressure, independent of the compounds used. At equivalent osmotic pressure, each compound [glycerol, dimethyl sulfoxide (DMSO), sucrose, and PEG 2000] produced an
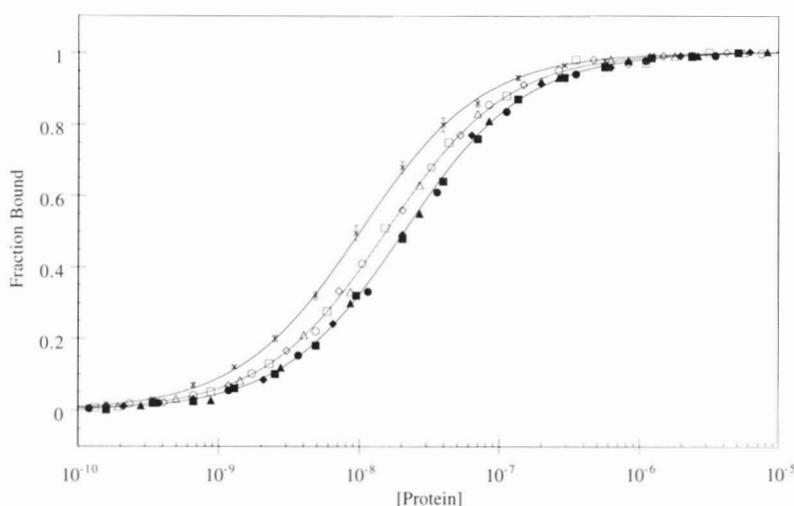
Fig. 2. HinL–*hixL* binding isotherms. Fraction DNA bound obtained from quantitative phosphorimager scans is plotted versus Hin peptide concentration. Data points indicated by symbols represent an average of at least five independent measurements: crosses, 0 atm osmotic pressure; open symbols, 50 atm osmotic pressure; filled symbols, 100 atm osmotic pressure. Data obtained using different compounds is indicated as follows: squares, DMSO; triangles, glycerol; diamonds, PEG; circles, sucrose. For clarity, not all data points are shown for each compound. Also for clarity, representative error bars are shown only for the 0 atm data, indicating one standard deviation. Errors for the 50 and 100 atm data are of similar magnitude as those shown. The solid lines indicate the best fits to Equation 1. For 50 and 100 atm data, combined data for all four compounds at a each osmotic pressure was fit as a single data set, and the solid line displayed represents the best fit to this aggregate data. The K_d values for the individual data sets are listed in Table 1. Fits of the binding isotherms for each data set are superimposable within the error of the measurement. Conditions are as indicated in the caption to Figure 1.

Table 1. HinL-*hixL* dissociation constants

Osmotic pressure	K_d (nM)[a]				
	Buffer[b]	DMSO	PEG 2000	Glycerol	Sucrose
0 atm	10.2 ± 0.5				
50 atm		15.6 ± 1.0	15.1 ± 0.9	15.5 ± 1.0	16.2 ± 1.2
100 atm		21.7 ± 0.8	22.4 ± 0.4	22.9 ± 0.7	22.1 ± 0.7

[a] Each value is the average of at least five independent experiments. Errors indicate one standard deviation.

[b] Standard buffer contained 25 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl, 100 μg/mL BSA, 0.02% Nonidet NP-40, and 10 μM sonicated salmon sperm DNA.

equivalent increase in the dissociation constant (Table 1). Osmotic pressure is the only colligative solvent property that correlates uniformly with binding—the concentrations of glycerol, DMSO, PEG 2000, and sucrose used here to induce 100 atm osmotic pressure produce relative viscosities that range from 1.4 to greater than 5, dielectric constants that range from 50 to 70, and water concentrations (excluded volumes) that range from 35 to 45 M (Robinson & Sligar, 1994).

The simplest explanation for this effect is that a net increase in water binding accompanies formation of the Hin–DNA complex. This result is surprising in that typically it is expected that formation of a macromolecular complex results in desolvation. Although it is undoubtedly true that portions of the protein and DNA surface are stripped of water, the results presented here argue strongly that a population of solvent molecules that are inaccessible to bulk solvent are present in the Hin–*hixL* complex that are not present in the free species. Thus, there appears to be a net increase in the number of waters associated with the complex, relative to the number associated with the free peptide and DNA.

The expression $\ln K_{eq} = \pi \Delta V/RT$ (where π is the osmotic pressure, ΔV is the change in volume, T is the temperature, and R is the gas constant) governs the relationship between the equilibrium constant and osmotic pressure (Rand et al., 1993). Assuming a constant volume for water of 18 mL/mol, we can roughly estimate the number of waters that bind when the Hin–*hixL* complex is formed. Of course, the actual molar volume of water can depend on surface interactions with the protein and the detailed hydrogen bonding states. From the magnitude of the measured increases in K_d at the two osmotic pressures, we can estimate a change in the number of waters bound of 10 ± 2. At present, we are unable to determine whether these water molecules are acting as discretely bound moieties in the protein–DNA interface (as observed in the crystal structure) or if they represent a population acting with more global effects on protein or DNA conformation (for example, altering the hydration of DNA favoring a shift from B- to A-form). However, it is clear that they must be inaccessible to the compounds added as osmolytes. Because no structural information is available regarding the number of waters associated with the free peptide or DNA, we can not compare this value directly with waters detected by crystallographic analysis.

Recently a thermodynamic fingerprint has been described for analysis of changes in heat capacity that indicate when folding transitions accompany protein–DNA binding (Spolar & Record, 1994). This phenomenon results from desolvation of the protein surface when folded. Bearing these results in mind, the observation of water binding coupled to formation of a protein–DNA

complex might indicate an *unfolding* transition that accompanies binding. Such a phenomenon has been observed in the binding of the *Bam*H I endonuclease to DNA (Newman et al., 1995).

Thus, our results are consistent with a model in which a few waters modulate binding through specific interactions with DNA bases and the protein backbone and side chains. However, it is important to recognize that these findings may reflect a more general role for water in which solvation and desolvation processes are associated with conformation changes that accompany binding. In either case, a crucial point of this paper is that researchers investigating molecular interactions must pay attention to the effects of solvent in considering the energetics of these systems.

In summary, we have shown conclusively that waters play a significant role in assisting the molecular recognition process between the Hin DNA-binding domain and its cognate DNA sequence, as suggested by the structure model developed in the X-ray crystallographic analysis. These findings indicate that, in addition to participating in sequence discrimination in restriction enzymes, an important role for water may exist in invertase–DNA interactions, and possibly in structurally similar DNA-binding proteins such as helix-turn-helix bacterial repressor proteins, and eukaryotic homeodomain proteins as well. Additionally, because we detect the participation of several water molecules in excess of the two observed in the crystal structure, it is likely that, in some systems, the interstitial waters observed in X-ray crystal structures represent some, but not necessarily all, of the functionally significant waters in these complexes.

This study shows that the osmotic stress approach can be used in many contexts to detect the coupling of water binding to molecular processes. Moreover, the phenomenon of water-assisted recognition may be quite general, and extend over a broad range of macromolecular systems.

As appreciation for the participation of bound waters in molecular recognition grows, a key question is why so many systems have evolved to use this additional partner in binding. One possibility is that the presence of water molecules allows for greater variety and flexibility for binding. For example, interfacial waters may allow a protein to bind to one sequence under "normal" conditions, and one or many alternative sequences under altered conditions (such as elevated osmotic pressure), with the presence or absence of waters acting as a switch for recognition specificity, as observed in the *Eco*R I and other restriction endonuclease systems (Robinson & Sligar, 1994, 1995a).

If waters are being used as a structural "switch," it is possible that binding to one or more alternate sequences will be enhanced by the release of water, which must accompany application of osmotic pressure (analogous to the phenomenon observed in re-

striction endonucleases). Future studies in the invertase, restriction enzyme, and transcription factor systems will no doubt further elucidate the mechanism of this novel role for water in molecular recognition.

Materials and methods: *Peptide and DNA synthesis:* The 52-amino acid peptide corresponding to the Hin recombinase DNA-binding domain (Feng et al., 1994) was synthesized by solid-phase synthetic methods by the Genetic Engineering Facility at the University of Illinois. The sequence of the peptide was identical to that used in the determination of the crystal structure. The peptide was purified using reversed-phase and ion-exchange liquid chromatography methods. The composition, molecular weight, and purity of the peptide were verified by amino acid analysis and electrospray mass spectroscopy.

A 26-base pair dsDNA fragment containing one-half site of the left half of the Hin recombination site (*hixL*) was constructed for use in DNA gel mobility shift assays. The sequence is:

5'-T-G-A-C-C-G-C-**G-T-T-T-T-T-G-A-T-A-A**-C-C-G-A-C-A-G-T-3'

3'-A-C-T-G-G-C-G-**C-A-A-A-A-A-C-T-A-T-T**-G-G-C-T-G-T-C-A-5',

where the half-site is indicated in bold type, and the flanking sequences are nonspecific DNA that was included to maintain the stability of the oligonucleotide cassette.

The oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer. Oligonucleotides were synthesized with the trityl group on, purified by reversed-phase HPLC, detritylated, and repurified by HPLC.

The top strand was end-labeled for use in gel shift assays using standard methods (Sambrook et al., 1989; Brown & Sauer, 1993). Briefly, the oligo was kinased using γ-^{32}P-labeled ATP and T4 polynucleotide kinase. The reaction was heat-inactivated, a twofold excess of the complimentary strand was added, and the strands were heated to 90 °C and slowly cooled to anneal. The mixture was extracted with phenol:chloriform, and passed over a Sephadex G-25 spin column to separate the labeled duplex from unincorporated nucleotide.

Gel shift assays: Equilibrium binding assays were performed essentially as described previously (Hughes et al., 1992; Brown & Sauer, 1993). Hin peptide was incubated with labeled *hixL* DNA at room temperature for 2 h. Binding buffer consisted of 25 mM Tris-Cl, pH 8.0, 5 mM MgCl$_2$, 0.1 mM EDTA, 100 mM NaCl, 100 μg/mL BSA, and 0.02% Nonidet NP-40; 10 μM sonicated salmon sperm DNA was added as a nonspecific competitor. The concentration of *hixL* DNA was 2 pM. Hin peptide concentrations ranged between 2×10^{-11} and 9×10^{-6} M and were always at least 10-fold higher than the concentration of the DNA.

A series of experiments was performed in which glycerol, DMSO, PEG 2000, or sucrose was added to induce osmotic pressure of 50 and 100 atm. The concentration of these cosolvents and solutes was approximately 1.2 M for π = 50 atm, and 2.5 M for π = 100 atm (Parsegian et al., 1995). Osmotic pressures were determined using vapor pressure osmometry as described previously (Robinson & Sligar, 1993). Values of osmotic pressure indicated are relative to that of the standard buffer.

Glycerol was added to <5% total volume just prior to loading the samples onto 10% polyacrylamide gels. No tracking dye was added to the samples, but a control lane containing only dye (bromophenol blue and xylene cynol) was included to monitor progress. Gels were run at 300 V while loading, and until the dye had migrated into the gel, at which point the voltage was reduced to 150 V. Gels were dried and exposed using phosphorimager screens (Molecular Dynamics), and band intensities quantitated with the Volume Measurement utility using ImageQuant software (Molecular Dynamics). Fraction of DNA bound was determined as the volume of the band corresponding to bound DNA divided by the sum of all bands.

Because Hin is always present in significant excess of the DNA, the concentration of free protein can be approximated closely by using the total protein concentration $[P_t]$. For Hin peptide binding to the *hixL* half-site, the binding isotherm simplifies to:

$$\Theta \approx \frac{1}{1 + \frac{K_d}{[P_t]}}, \tag{1}$$

where Θ is the fraction of bound DNA, and K_d is the equilibrium dissociation constant,

$$K_d = \frac{[P][\text{DNA}]}{[P \cdot \text{DNA}]}. \tag{2}$$

Thus, the K_d is equal to the concentration of Hin peptide at half maximal binding. Data were fit to Equation 1 by nonlinear least-squares analysis using the program Kaleidagraph (Abelbeck Software) running on a Macintosh computer.

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