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Water release associated with specific binding of *gal* repressor

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Water release coupled to the association of *gal* repressor with DNA is measured from the sensitivity of the binding constant to the solution osmotic pressure, using neutral solutes that are typically excluded from polar protein and DNA surfaces. Differences in water release for binding of repressor to different sequences are linked with differences in specificity and binding energies. With sucrose, the specific binding of repressor to operator sequences is accompanied by the release of 130 water molecules. No water release is seen for the weak, non-specific binding of repressor to poly(dI-dC)·(dI-dC). A difference in the release of six water molecules is seen even for the binding of *gal* repressor to two different operator sequences that differ in affinity by only a factor of two.

Key words: *gal* repressor/hydration/osmotic stress/protein–DNA interactions

Introduction

The functioning of many gene regulatory proteins requires an ability to recognize specific DNA sequences and to bind with high affinity. Crystal structures of protein–oligonucleotide complexes show extensive, stereospecific networks of hydrogen bonds and salt bridges between amino acid side chains and the DNA backbone and grooves (e.g. Steitz, 1990; Pabo and Sauer, 1992). In addition, the thermodynamics of numerous specific protein–DNA complexes have been studied in some detail (e.g. Mossing and Record, 1985; Brenowitz *et al.*, 1990; Takeda *et al.*, 1992; Spolar and Record, 1994; Ladbury *et al.*, 1994). In spite of this wealth of information, however, the linkage between the thermodynamics of binding strength and structural specificity is not yet well established. There does not seem to be a simple additivity connecting individual molecular interactions and total binding free energy (e.g. Mossing and Record, 1985; Record and Spolar, 1990; Lesser *et al.*, 1990). Recent results indicating strong water structuring forces between many polar macromolecules (Leikin *et al.*, 1993) suggest that the change in hydration associated with binding could be a key thermodynamic variable for dissecting protein–DNA interactions. The close association of complementary protein and DNA surfaces, of necessity, displaces many surface bound water molecules. These are waters that can be probed by the osmotic stress technique (Zimmerberg and Parsegian, 1986; Kornblatt and Hoa, 1990; Colombo *et al.*, 1992; Rand *et al.*, 1993), using neutral solutes that are typically excluded from the hydration waters surrounding polar protein and DNA surfaces (Timasheff, 1993). Just as the dependence of binding constants on temperature gives an entropy, or on pH gives a number of released protons, or on salt activity gives a change in the number of bound ions, the sensitivity of repressor binding constants to water activity gives the number of solute-excluding water molecules released in the reaction.

The *gal* operon of *Escherichia coli* is negatively controlled by the binding of *gal* repressor to two operator sites, O_E and O_I. Repressor binding to both operators is necessary for repression, suggesting DNA looping stabilized by protein–protein interactions (Adhya, 1989). Repressor is thought to bind to each operator as a preformed dimer (Brenowitz *et al.*, 1990). We show here that the binding of *gal* repressor to its operator sequences is dependent on the concentration of the neutral solutes, betaine glycine, sucrose and triethylene glycol. Binding energies vary linearly with water chemical potential or, equivalently, osmotic pressure. The inferred number of solute-excluding water molecules released with protein binding is only secondarily sensitive to the solute's chemical nature. With sucrose, ~130 waters are released on the binding of repressor to operator DNA. Binding of repressor to non-specific poly(dI-dC)·(dI-dC), however, has no osmotic sensitivity. No water is released with non-specific binding. Water release seems to provide a link between structure and thermodynamics that is now missing. It is, of course, measured as a thermodynamic parameter coupling binding energy and bulk water activity. On another level, it is also a structural parameter related to the closeness of contact between DNA and protein surfaces and to possible conformational changes or interactions of proteins.

Typically, biochemical reactions are studied in dilute buffer solution with minimal added salt, using purified proteins and nucleic acid fragments. Unlike a dilute solution, however, the interior of a eukaryotic or prokaryotic cell contains high concentrations of macromolecules (>300 mg/ml), much of which is highly organized in cytoskeletal structures and in multi-enzyme complexes. The large effects of this macromolecular crowding (or stress) and polymer-excluding volume on reactions within a cell have been well established (Garner and Burg, 1994; Zimmerman and Minton, 1993). Both prokaryotic and eukaryotic cells also accumulate significant concentrations of small, neutral osmolytes to balance the external osmolality. The large effects on repressor binding reported here are seen with concentra-

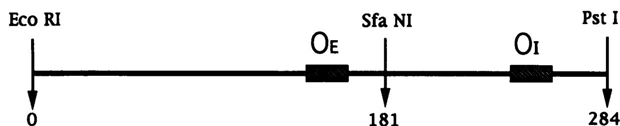

Fig. 1. Schematic diagram of the *Eco*RI−*Pst*I fragment of plasmid pSA509. The isolated fragment was used either as shown (two-site fragment) or cleaved with *Sfa*NI, to generate fragments bearing either O_E (181 bp) or O_I (103 bp).

tions of solutes similar to those often seen *in vivo*. This observation of the release of many water molecules associated with repressor binding then also has important consequences for function under the conditions of 'osmotic crowding' (or stress) and solute-excluding water found within cells.

Results

Water release accompanies the specific binding of gal repressor to operator

The DNA fragments containing the *gal* operator sequences, O_E and O_I, used here are illustrated in Figure 1. The 284 bp DNA fragment contains both operator sequences in the natural arrangement of *gal* repressor binding sites found in the *gal* operon of *E.coli* (Majumdar and Adhya, 1984). This fragment is cut with *Sfa*NI to generate fragments with separate O_E and O_I operators.

The increase in *gal* repressor binding to the DNA fragment containing both the O_E and O_I operators that is observed with increasing sucrose or triethylene glycol (EG_{150}) concentration is shown in Figure 2. The increase in *gal* repressor affinity is qualitatively very similar for these two solutes, in spite of their very different chemical natures. The effect of neutral solutes on repressor binding can be quantitated by the gel mobility shift assay, which has previously been shown to give accurate results for *gal* repressor binding (Brenowitz *et al.*, 1990; Senear and Brenowitz, 1991). Pseudo binding constants can be defined from the relative fractions of DNA with no bound repressor, with one bound and with two bound, F_0, F_1 and F_2, respectively, for the association of the first repressor ($K_1 = F_1/F_0$) and for the association of the second ($K_2 = F_2/F_1$). Since we cannot distinguish between binding to O_E or O_I, K_1 is a simple average of the two elementary binding constants, K_{OE} and K_{OI}; K_2 is a more complicated average that will also include contributions from any cooperative (or anti-cooperative) interaction between sites. The average binding constant of first repressor to operator is $\sim 3\times10^8\ M^{-1}$, in agreement with binding constants previously measured by footprinting (Brenowitz *et al.*, 1990) under similar conditions.

The dependence of the free energy for binding the first and second repressors on neutral solute concentration is shown in Figure 3 for three neutral solutes (sucrose, betaine and triethylene glycol) and with four repressor concentrations. For each solute, binding free energies associated with both K_1 and K_2 increase linearly with the osmolal concentration of solute. This linearity is consistent with an exclusion of these solutes from the water surrounding the associating protein and DNA surfaces. The difference in solute concentration in the close vicinity of the protein and DNA and in the bulk solution defines an

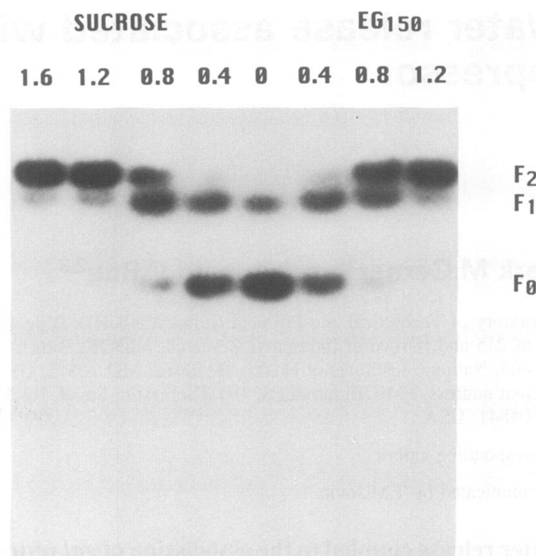

Fig. 2. Neutral solutes dramatically enhance the binding of *gal* repressor to operator. An autoradiogram from a gel-shift experiment shows the change in *gal* repressor binding to the two-operator fragment with change in concentration of two neutral solutes, sucrose and triethylene glycol (EG_{150}). DNA fragment concentration was 0.1 nM, with 1 nM *gal* repressor. Reaction conditions and electrophoresis were as described in Materials and methods. The position of the free DNA is shown by F_0, DNA with one repressor bound by F_1, and with two repressors bound by F_2. Sucrose or EG_{150} concentration (in molal) are as indicated. Similar results were observed at 4°C.

osmotic stress that favors the release of solute-excluding water accompanying the close association of repressor and operator. The magnitude of the effect depends both on the solute osmolal concentration (equivalent to the bulk water chemical potential) and on the difference in the number of solute-excluding waters associated with the complex and the number associated with the free repressor and DNA. This osmotic effect can be quantitated analogously to the conventional analysis of the effect of salt activity on repressor binding, i.e. the slope of $\log(K_b)$ versus $\ln(a_i)$, where a_i is the salt activity, is 2.303 Δn_i, where Δn_i is the change in the number of bound ions coupled to protein binding (Record and Spolar, 1990). Similarly, the slope of $\log(K_b)$ versus $\ln(a_w)$, where a_w is the water activity, is 2.303 Δn_w, where Δn_w is the change in the number of solute-excluding water molecules coupled to repressor binding. Since $\ln(a_w) = -[\text{solute}]_{\text{osmolal}}/55.5$, where $[\text{solute}]_{\text{osmolal}}$ is the osmolal concentration of solute and 55.5 is the number of moles of water in 1 kg, the slope of the lines shown in Figure 3 is given by,

$$\frac{d\ \log(K_b)}{d[\text{solute}]_{\text{osmolal}}} = -\frac{2.303\ \Delta n_w}{55.5}$$

For each solute, no apparent dependence of Δn_w on *gal* repressor concentration between 0.5 and 4 nM nominal dimer concentration is seen. Additionally, no significant difference in the osmotic sensitivity is observed between binding the first repressor and binding the second. A somewhat different number of released solute-excluding water molecules is seen with each solute, with Δn_w = 100 ± 12 for betaine, 130 ± 15 for sucrose and 180 ± 15 for triethylene glycol.

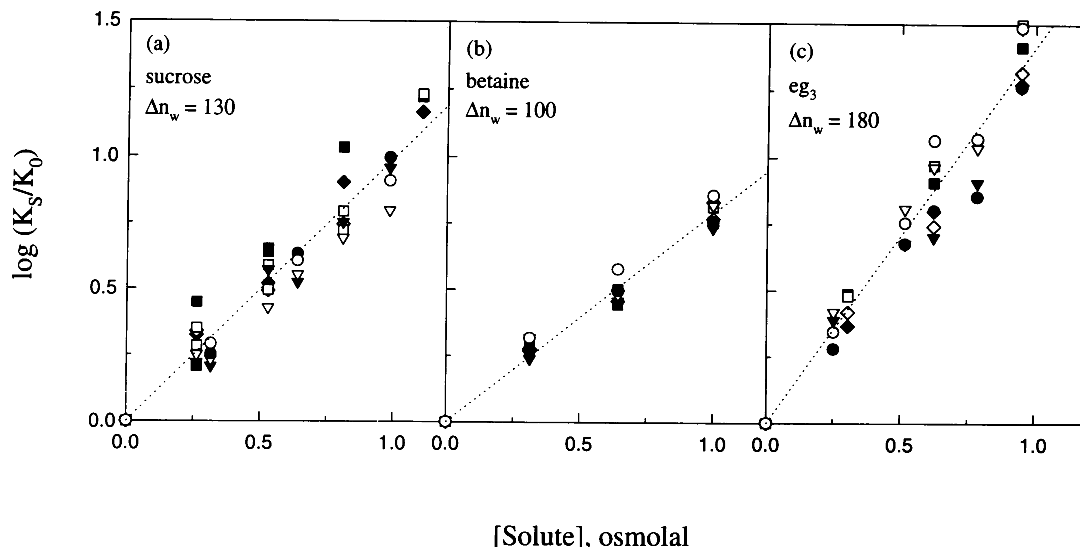

Fig. 3. The osmotic sensitivity of *gal* repressor binding to the two-operator fragment. The fraction of free DNA and DNA complexes with one or two bound repressors was extracted from gels such as that shown in Figure 2 using a Molecular Dynamics PhosphorImager. The resulting data are shown here plotted as the ratio of the two operator DNA fragment fraction with two bound repressors to the fraction with one bound, log (F_2/F_1) (closed symbols), and of the one bound to the free DNA fractions, log (F_1/F_0) (open symbols) versus the solute osmolal concentration. Data are shown for 0.5 nM (○), 1 nM (▽), 2 nM (□) and 4 nM (◇) *gal* repressor concentration. Binding constants are normalized by the appropriate values with no added solute, log (K_S/K_0), for ease of comparison. The dotted lines are the best linear fit to the data. The values of Δn_w are calculated from the slopes as described in the text. The average error is ~10%.

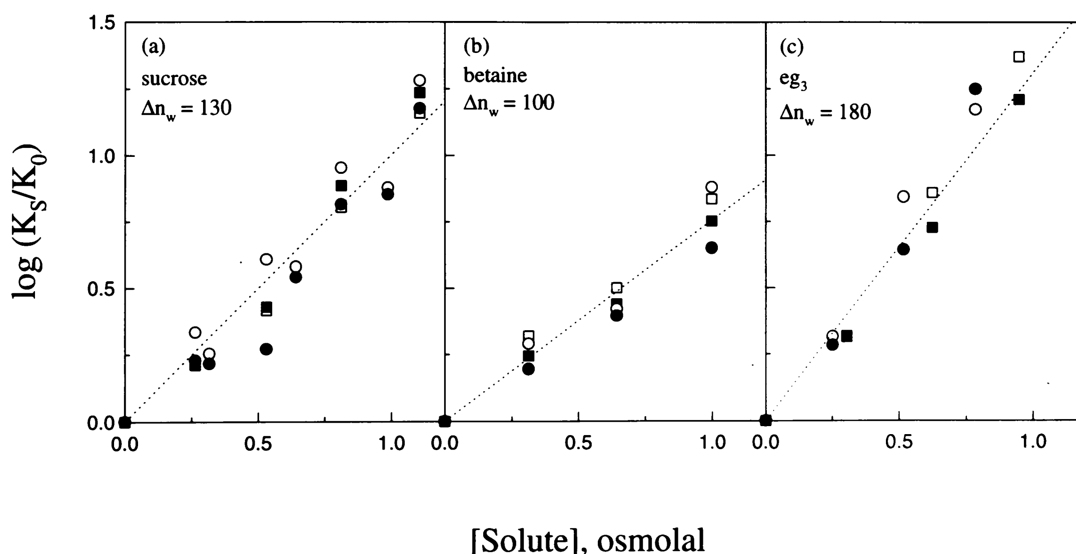

Fig. 4. The osmotic sensitivity of *gal* repressor binding to the one-operator fragments. Conditions are the same as in Figure 3, except that the two fragments containing separate O_E or O_I operators, generated from *Sfa*NI digestion of the two operator fragment, were used. The difference in labeling efficiencies allow us to identify the bands corresponding to repressor bound O_E and O_I complexes. The ratio of repressor bound fragment to free DNA for the separate O_E (open symbols) and O_I (closed symbols) operators is shown as a function of the solute osmolality for 0.5 nM (○) and 1 nM (□) repressor. As in Figure 3, equilibrium binding constants are normalized by the appropriate values without added osmolyte.

The osmotic sensitivity of repressor binding to the separate O_E and O_I operators is shown in Figure 4 for the three solutes and with two repressor concentrations. The binding constant to the O_E operator is ~2-fold larger than that to the O_I site, also in agreement with previous measurements (Brenowitz *et al.*, 1990). The apparent number of water molecules released on repressor binding, Δn_w, is virtually indistinguishable from that for the binding of repressor to the fragment containing both operators (Figure 3).

No water release accompanies non-specific binding of gal repressor

This apparent release of water is directly pertinent to the thermodynamics of specific recognition if Δn_w is shown to be sequence dependent. The difference in osmotic sensitivities between operator and non-specific DNA binding can be probed by direct competition. The inset of Figure 5 shows the loss of repressor binding to the separate O_E and O_I sites with increasing poly d(I-C)·d(I-C) concentration. As described in Materials and methods and

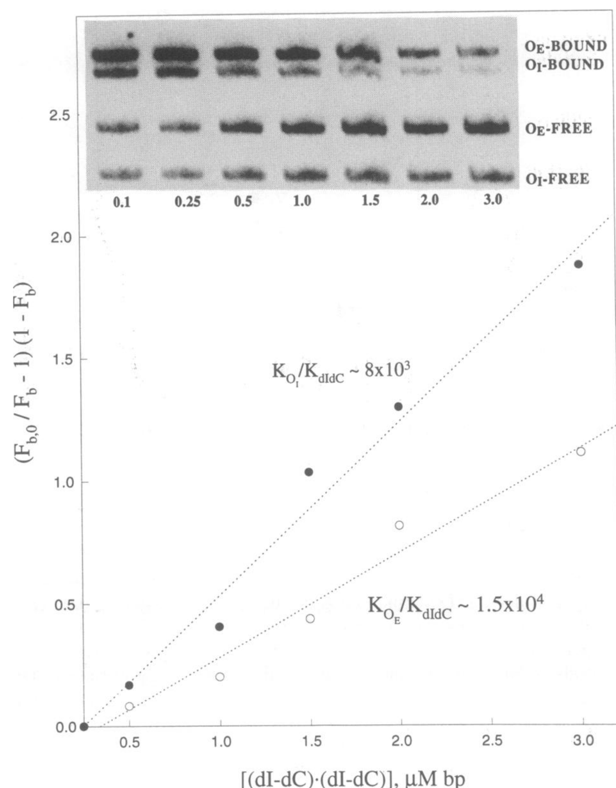

Fig. 5. The competition for repressor binding between operator DNA and poly(dI-dC)·(dI-dC). Inset: the gel mobility shift assay shows the decrease in specific repressor binding to the O_E and O_I containing fragments with increasing concentration of poly(dI-dC)·(dI-dC). Stoichiometric repressor binding reaction conditions are used as described in Materials and methods. Total DNA fragment concentration was 0.4 nM, with 0.25 nM *gal* repressor concentration. Main figure: the loss of specific binding as a function of competitor was quantitated as is also described in Materials and methods. F_b is the fraction of fragment with bound repressor at different poly(dI-dC)·(dI-dC) concentrations and $F_{b,0}$ is the fraction bound with 0.25 µM bp poly(dI-dC)·(dI-dC). The slope is used to calculate the ratio of specific to non-specific binding constants for the O_E (○) and O_I (●) operators.

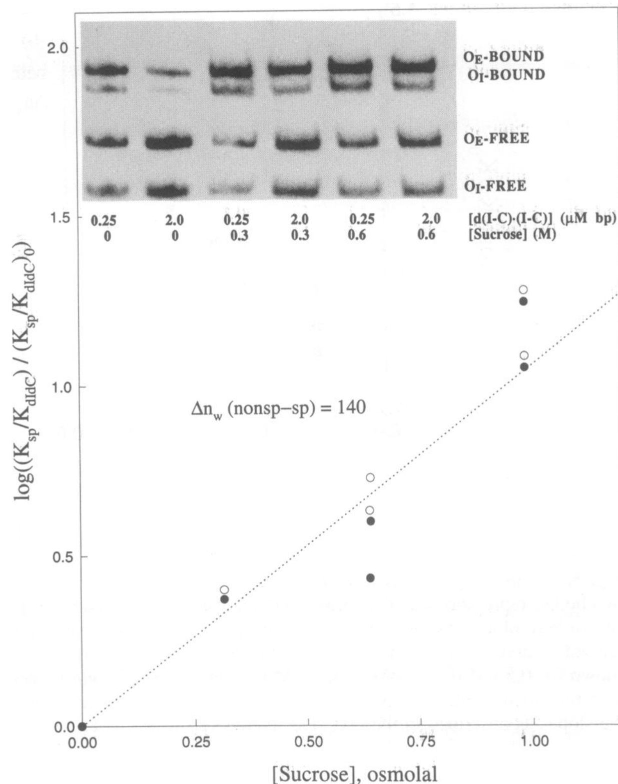

Fig. 6. The osmotic sensitivity of the competition between operator and poly(dI-dC)·(dI-dC) for repressor binding. Inset: The gel mobility shift assay shows the increase in specific binding with increasing sucrose concentration. Reaction conditions and fragment and repressor concentrations were the same as in Figure 5 and with the indicated poly(dI-dC)·(dI-dC) and sucrose concentrations. Main figure: the osmotic sensitivity of the ratio of specific to non-specific binding constants is quantitated for the O_E (○) and O_I (●) operators. Binding constant ratios are normalized by their values with no added sucrose. The dotted line is the best fit for the osmotic dependence of binding repressor from bulk solution from Figures 3 and 4.

shown in Figure 5, under conditions of virtually stoichiometric repressor binding, the ratio of specific to non-specific binding constants can be extracted from this loss of specific operator–repressor complex with increasing competitor DNA concentration. The 2-fold difference in binding constants for the O_E and O_I operator sites seen in titration experiments is reflected in the 2-fold difference in competitive binding constants.

As seen in the inset of Figure 6, poly d(I-C)·d(I-C) is not able to compete as well for repressor binding with increasing sucrose concentration. Under these conditions of stoichiometric repressor binding, increasing sucrose concentration has no effect on the total amount of specific operator–repressor complex (O_E + O_I) in the absence of poly d(I-C)·d(I-C). The effect of sucrose on poly d(I-C)·d(I-C) competition is, therefore, not due to an increase in active protein concentration, but rather to a decrease in the ratio of non-specific to operator binding constants. The osmotic dependence of the specific–non-specific binding free energy difference is shown in Figure 6. The 140 ± 25 water molecules released in transferring a repressor from competitor poly d(I-C)·d(I-C) to an operator site is not significantly different from that seen in Figures 3 and 4 for the transfer from bulk solution to operator (130 waters).

The osmotic sensitivity of the competition between O_E and O_I sites

Although, under stoichiometric binding conditions, there is no change in the total fraction of operator–repressor complex with increasing sucrose concentration in the absence of competitor poly d(I-C)·d(I-C), a small shift in repressor binding from the O_I to the O_E operator site is seen. This shift represents a change in the ratio of operator binding constants, K_{OE}/K_{OI}. Figure 7 shows the osmotic sensitivity of the binding free energy difference between the O_I and O_E sites. The more weakly bound O_I site releases some 6 ± 3 fewer waters than the O_E site.

Discussion

The osmotic stress technique has been used previously to probe changes in number of waters associated with the opening and closing of membrane bound channels (Zimmerberg and Parsegian, 1986), with the electron transfer reaction of cytochrome oxidase (Kornblatt and Hoa, 1990), with the oxygenation of hemoglobin (Colombo

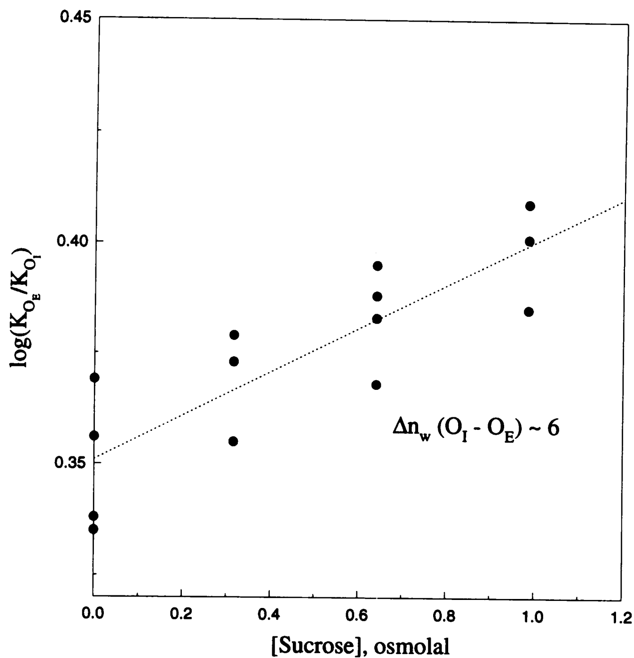

Fig. 7. The osmotic sensitivity of the competition between O_E and O_I operators for repressor binding. The difference in binding free energy between the O_I and O_E sites is shown as a function of sucrose osmolality. Reaction conditions are the same as in Figure 5, with 0.25 μM bp poly(dI-dC)·(dI-dC) added. Binding constant ratios are calculated as described in Materials and methods. The dotted line is the best fit to the data, which gives $\Delta n_w = 6 \pm 3$ for transferring a repressor from the O_E to the O_I site.

et al., 1992) and with the binding of glucose by hexokinase (Rand *et al.*, 1993). The technique relies on an exclusion of solute from water associated with proteins or macromolecular complexes and a difference in the number of solute-excluding waters between two conformations of the protein or complex. In its most straightforward application, solute is sterically excluded from water filled cavities or pores. The difference in solute concentration in the bulk solution and in these cavitities is thermodynamically equivalent to an osmotic pressure that acts to favor those conformations with smaller cavities or pores, i.e. that release water.

Applied to the *gal* repressor–DNA binding reaction, the osmotic stress technique would then probe the difference in the amount of solute-excluding water between the complex and the free repressor and DNA. In contrast to an ideal steric exclusion of solute, however, the complementary surfaces of the free repressor and operator DNA are well exposed to the bulk solution. There are no well defined spaces inaccessible to the solutes used here. Even in this case, however, there is ample evidence that many solutes are still highly excluded from native protein surfaces. Direct measurement of solute–protein interactions through solution density changes shows that many native proteins are 'preferentially hydrated', excluding solutes as betaine and sucrose from the water in close contact with the protein surface (reviewed in Timasheff, 1993). The energies of exclusion for solutes like sucrose scale approximately linearly with solute concentration (Lee and Timasheff, 1981). Energies associated with strongly excluded solutes also seem to scale with exposed protein surface area (Arakawa and Timasheff, 1982). When measured for the same system, exclusion energies for sugars and betaine are similar (Arakawa and Timasheff, 1982, 1983). In almost all respects, the exclusion of solutes such as sucrose and betaine from exposed protein surfaces through 'preferential hydration' closely parallels steric exclusion. The amount of solute-excluding water associated with exposed surfaces, however, will depend somewhat on the chemical natures of both the solute and the macromolecular surface and, therefore, can not be as straightforwardly interpreted as with sterically excluded solutes.

Figures 3 and 4 show that binding energies of *gal* repressor to operator sequences are linearly dependent on water chemical potential or, equivalently, solution osmotic pressure. This increased binding with increasing solute concentration is not due to changes in the solution dielectric constant and its consequent effect on electrostatic energies. Whereas a 1 M betaine solution increases the dielectric constant from 80 to ~92 at 20°C (Edsall and Wyman, 1958), 1 M solutions of both sucrose and triethylene glycol decrease it to ~72 (Malmberg and Maryott, 1950; Feakins and French, 1957); yet all three solutes show an increase in repressor binding.

The linear dependence of binding energy on solute concentration is most reasonably explained by a linkage between repressor binding and water release. Not unexpectedly, the net release of solute-excluding water accompanying repressor–operator complex formation, Δn_w, inferred from the slopes in Figures 3 and 4 varies somewhat with the chemical nature of the solute, 100 for betaine, 130 for sucrose and 180 for triethylene glycol. If the effect was the result of very weak direct solute binding, then solute binding constants and the number of solute binding sites would have to be implausibly similar for these very different solutes.

It is somewhat surprising that the apparent water release is constant over all the conditions examined in Figures 3 and 4. Although a *gal* repressor dimer dissociation constant of 2 nM has been inferred previously from fits of titration data (Brenowitz *et al.*, 1990), no dependence of Δn_w on the nominal repressor dimer concentration between 0.5 and 4 nM is observed. Either the dimerizing surfaces of the protein do not exclude these solutes (the net release of water associated with dimerization is negligible compared with direct repressor–operator binding, perhaps due to compensating surface area changes in other regions of the protein that accompany dimerization), or the indirectly extracted dimer dissociation constant is artifactual and the real constant is much smaller.

The data in Figures 3 and 4 also show the same net solute-excluding water release for the binding of repressor to the separated operators and for the binding of the first and second repressors to the fragment containing both operators. Genetic experiments have demonstrated that DNA loop formation mediated by operator bound *gal* repressors occurs *in vivo* (Adhya, 1989). Previous direct repressor titration data (Brenowitz *et al.*, 1990; Senear and Brenowitz, 1991), however, showed no *in vitro* cooperative binding of the second repressor to the two operator fragment indicative of a protein–protein interaction. Now the osmotic stress experiments show no additional release of water for binding the second repressor, also suggesting no significant interaction between bound repressors *in vitro*.

Although no crystal structure is available for the *gal* repressor complex, in analogy to other helix−turn−helix motif DNA binding proteins, repressor−operator interactions are likely predominately direct, displacing the interactions of the surfaces with hydrating water when free in solution. Neglecting additional protein conformational changes that may be linked with binding, the change in the number of solute-excluding water molecules coupled to repressor binding will be proportional to the solvent inaccessible surface area of the protein−DNA interface. If we assume that both *gal* and *trp* repressors are reasonably typical helix−turn−helix motif DNA binding proteins with similar extents of contact between DNA and protein, then the ~29 nm^2 area measured for the *trp* repressor/operator complex (Otwinowski *et al.*, 1988; Lawson and Carey, 1993) translates into an estimated release of 240–300 water molecules (first water layer) for the binding of *gal* repressor. About half this water would then seem to interact strongly enough with protein or DNA groups to exclude solutes. The heat capacity associated with *gal* repressor binding (Brenowitz *et al.*, 1990) may, however, indicate that additional surface area is buried in a protein conformational change upon binding (Spolar and Record, 1994). Preliminary osmotic stress experiments on the binding of CRP protein to its site on the two *gal* operator fragments (data not shown) indicates that water release accompanying specific binding is probably a general phenomenon. The enhancement of CRP−cAMP binding with increasing osmotic pressure is qualitatively similar to that observed for *gal* repressor.

The large number of water molecules released with binding of repressor to operator reflects the large surface area of direct contact between protein and DNA. The number of waters released is as much a structural parameter, defining an extent of close interaction, as it is a thermodynamic one. The difference in Δn_w between operator and non-specific binding defines the difference in the extent of close protein−DNA association and is, therefore, directly relevant to the general problem of specific recognition (von Hippel, 1994). Although specific binding constants are quite sensitive to salt concentration through a release of DNA counterions, for example, a similar (or sometimes even greater) salt sensitivity is also typically observed for non-specific binding (Record and Spolar, 1990). Although salt release provides binding stability, it is not coupled to specificity. In contrast, Figure 6 shows that the release of water accompanying the transfer of repressor from bulk solution to operator complex is the same, within experimental error, as the transfer of repressor from a non-specific association with poly d(I-C)·d(I-C) to operator complex. The binding of free repressor to poly d(I-C)·d(I-C) releases no water. The non-specific interaction of *gal* repressor with poly d(I-C)·d(I-C) seems not to involve direct contacts. Both protein and DNA essentially retain their full complement of solute-excluding waters of hydration in the non-specific complex. Analogous to solute exclusion, repressor and DNA are themselves also excluded from each other's hydrating waters, until the correct match with operator can be made.

A connection between strength of interaction and water release even extends to the comparatively small factor of two differences in repressor binding constants between the O_E and O_I sites that is accompanied by a change of 6 (± 3) bound waters (Figure 7). These extra waters may be similar to those seen in some crystal structures (e.g. Otwinowski *et al.*, 1988; Lawson and Carey, 1993) mediating protein−DNA contacts, perhaps used as adaptors between the protein surface and non-optimal DNA sequences.

The direct contribution of these displaced water molecules to specific binding energetics is not clear. It is axiomatic that the interaction energy of binding is the difference between the direct protein−DNA interactions in the complex and the water−protein and water−DNA interactions of the free components. The waters seen released with specific repressor binding are bound sufficiently strongly by the protein and DNA surfaces to exclude neutral solutes. Additionally, release of the large number of water molecules observed with specific repressor binding must contribute a large positive entropy to the free energy.

More fundamentally, direct force measurements on condensed arrays of systems as diverse as DNA helices, lipid bilayers, collagen triple helices and carbohydrates demonstrate the apparent ubiquity and dominance of water structuring interactions between polar surfaces at close distance (reviewed in Leikin *et al.*, 1993). These hydration forces are much stronger than electrostatic or van der Waals interactions conventionally considered important. Surfaces can be strongly repulsive or equally strongly attractive depending sensitively on the complementarity of the surfaces. In general, as two surfaces come together interaction energies associated with hydration forces increase exponentially with number of waters displaced. The direct force measurements condensed arrays strongly suggest that the binding energetics of repressor to different DNA sequences in dilute solution should be strongly correlated with Δn_w.

Although the presence of high concentrations of neutral solutes may seem non-biological, such conditions in fact approximate the physiological milieu more closely than do the simple buffered salt solutions typically used. Both prokaryotic and eukaryotic cells accumulate significant concentrations of neutral osmolytes. In cells which must adapt to high external osmolality, the concentration of these 'compatible osmolytes' can attain the molar range under conditions of cellular osmotic stress (Cayley *et al.*, 1991; Garcia-Perez and Burg, 1991; Garner and Burg, 1994). As we have shown here, these compatible solutes are not inert. The presence of these solutes at high concentrations has dramatic effects on the extent of specific protein−DNA association. In addition to the well studied polymer-excluded volume effect (Zimmerman and Minton, 1993), osmotic stress effects on DNA−protein interactions will also contribute significantly to the stability of DNA−protein interactions *in vivo*.

Materials and methods

DNA fragments and protein

The 284 bp *Eco*RI−*Pst*I fragment of the plasmid pSA509 (Majumdar and Adhya, 1984), which contains the two wild type *gal* operator sites, O_E and O_I ,was isolated from an LE agarose gel by continuous elution from a trough cut into the gel just in front of the band, phenol/chloroform extracted and ethanol precipitated. After resuspension, the fragment was further purified using a NACS (Life Technologies, Inc.) column. The two operator fragment was digested with *Sfa*NI to generate the fragments

with separate O_E (181 bp) and O_I (103 bp) sites. After treating with calf intestinal phosphatase, the fragments were end-labeled using the forward reaction of T4 polynucleotide kinase and [γ-^{32}P]ATP. Repressor, isolated and stored as described in Majumdar *et al.* (1987), was a kind gift of Dr Sankar Adhya. Poly(dI-dC)·(dI-dC) was purchased from Pharmacia and used without further purification.

Gel mobility shift binding assay and osmotic stress

Two solution conditions were used for repressor binding reactions. For normal titrations, DNA fragments at ~0.1 nM concentration were incubated for 45 min with *gal* repressor at 20°C in 140 mM KCl, 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 50 μg/ml acetylated BSA, 0.2 μg/ml chicken erythrocyte DNA and varied concentrations of betaine glycine, sucrose or triethylene glycol. Conditions for stoichiometric binding of repressor used for measurement of binding constant ratios between operator and non-specific poly(dI-dC)·(dI-dC) and between O_E and O_I operators were the same as above but with 0.1% Nonidet-P40 added and with the chicken erythrocyte DNA replaced by varied concentrations of poly(dI-dC)·(dI-dC). Total operator DNA fragment concentration was 0.4 nM, with a 0.25 nM *gal* repressor concentration.

Free DNA and repressor–DNA complexes were separated on a 5% polyacrylamide gel (3% bis/mono) in 40 mM Tris acetate (pH 7.0), 1 mM EDTA, thermostatted at 20°C, electrophoresing for ~2 h at 6 v/cm. Gels were dried under vacuum and exposed to X-OMAT AR film at −70°C with one LightningPlus Intensifying screen. Quantitative results for repressor binding were obtained by scanning the gel using a Molecular Dynamics PhosphorImager. Several different methods were used to analyze the total ^{32}P in each component band and found not to affect the final results significantly.

Solute osmolalities were measured using a Wescor model 5100C vapor pressure osmometer. Even at the highest solute concentrations used for quantitating the osmotic effect, measured osmolalities are not significantly different from ordinary molal concentrations, for example, 1 M solutions of sucrose, betaine and triethylene glycol correspond to 1.11, 1.12 and 1.06 osmolal, respectively. Salt and solute osmolalities are additive to within 10%. Within the range of concentrations used, these solutes act almost thermodynamically ideal.

Analysis of competitive binding data

The ratio of binding constants extracted from the competition between two sites, 1 and 2, for repressor binding is given by,

$$\frac{K_1}{K_2} = \frac{F_{b,1}(1-F_{b,2})}{(1-F_{b,1})F_{b,2}}.$$

where $F_{b,1}$ and $F_{b,2}$ are the fractions of DNA sites 1 and 2, respectively, with bound repressor ($1-F_b$ is the fraction free). This is the expression used for the ratio of binding constants between the O_E and O_I sites. For the competition between operator and poly(dI-dC)·(dI-dC), the fraction of non-specific sites with bound repressor can be estimated indirectly, analogous to (Fried, 1989). Assuming that the binding constant to non-specific DNA, K_{non}, is much smaller than for operator binding, K_O, the concentration of free non-specific DNA is essentially the total poly(dI-dC)·(dI-dC) concentration, C_{non}. Under conditions of stoichiometric repressor binding, the concentration of non-specific sites with bound repressor is the concentration of operator bound repressor lost due to added non-specific DNA, $(F^0_{b,O}-F_{b,O})\,C_O$, where $F^0_{b,O}$ is the fraction operator with bound repressor either without added poly(dI-dC)·(dI-dC) or with a small reference concentration of poly(dI-dC)·(dI-dC) and C_O is the concentration of operator sites. The expression for the ratio of operator and non-specific binding constants is then,

$$\frac{K_O}{K_{non}} = \frac{F_{b,O}}{(1-F_{b,O})(F^0_{b,O}-F_{b,O})}\,\frac{C_{non}}{C_O}$$

The plots shown in Figure 5 are based on a variant of this equation,

$$(1-F_{b,O})\left(\frac{F^0_{b,O}}{F_{b,O}}-1\right) = \frac{K_{non}}{K_O C_O}\,C_{non}$$

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