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 interactions between DNA 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_L. 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 osmolarity. The large effects on repressor binding reported here are seen with concentra-
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, OE and O1, 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 SfoNI to generate fragments with separate OE and O1 operators.

The increase in gal repressor binding to the DNA fragment containing both the OE and O1 operators that is observed with increasing sucrose or triethylene glycol (EG150) 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, F0, F1, and F2, respectively, for the association of the first repressor (K1 = F1/F0) and for the association of the second (K2 = F2/F1). Since we cannot distinguish between binding to OE or O1, K1 is a simple average of the two elementary binding constants, KOE and KO1; K2 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 \( \sim 3 \times 10^6 \text{ M}^{-1} \), 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 K1 and K2 increase linearly with the osmotic 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 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 osmolar 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(K1) versus ln(a1), where a1 is the salt activity, is 2.303 \( \Delta n_w \), where \( \Delta n_w \) is the change in the number of bound ions coupled to protein binding (Record and Spolar, 1990). Similarly, the slope of log(K2) versus ln(a1), where a1 is the water activity, is 2.303 \( \Delta n_w \) where \( \Delta n_w \) is the change in the number of solute-excluding water molecules coupled to repressor binding. Since \( \ln(a_1) = -[\text{solute}]_{\text{osmolal}}/55.5 \), where [solute]_{osmolal} is the osmolar 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,

\[
\frac{d \log(K_1)}{d[\text{solute}]_{\text{osmolal}}} = \frac{2.303 \Delta n_w}{55.5}
\]

For each solute, no apparent dependence of \( \Delta n_w \) 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 \( \Delta n_w = 100 \pm 12 \) for betaine, 130 \( \pm 15 \) for sucrose and 180 \( \pm 15 \) for triethylene glycol.
Water release upon protein–DNA complex formation

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 Phosphorlmager. 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 \((F_3/F_0)\) (open symbols) versus the solute osmolality. 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 \((K_3/K_0)\), for ease of comparison. The dotted lines are the best linear fit to the data. The values of \(\Delta n_w\) 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 O₂ and O₃ operators, generated from SfaNI digestion of the two operator fragment, were used. The difference in labeling efficiencies allow us to identify the bands corresponding to repressor bound O₂ and O₃ complexes. The ratio of repressor bound fragment to free DNA for the separate O₂ (open symbols) and O₃ (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 O₂ and O₃ operators is shown in Figure 4 for the three solutes and with two repressor concentrations. The binding constant to the O₂ operator is 2-fold larger than that to the O₃ site, also in agreement with previous measurements (Brenowitz et al., 1990). The apparent number of water molecules released on repressor binding, \(\Delta n_w\), 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 \(\Delta n_w\) 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 O₂ and O₃ 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_s is the fraction of fragment with bound repressor at different poly(dI-dC)-(dI-dC) concentrations and F_b0 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.

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_{O_E}/K_{O_I}. 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
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, $\Delta n_w$, 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 $\Delta n_w$ 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 artificial 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 \(-29 \text{ nm}^2\) area measured for the trp repressor/operator complex (Otwinski 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 \(\Delta n_w\) 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\(_R\) and O\(_L\) 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. Otwinski 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 \(\Delta n_w\).

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 EcoRI–PstI fragment of the plasmid pSA509 (Majumdar and Adhya, 1984), which contains the two wild type gal operator sites, O\(_R\) and O\(_L\), 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 SfaNI to generate the fragments.
with separate $O_2$ (181 bp) and $O_3$ (103 bp) sites. After treating with calf intestinal phosphatase, the fragments were end-labeled using the forward reaction of T4 polynucleotide kinase and [γ-32P]ATP. Repressor, isolated and stored as described in Majumdar et al. (1987), was a kind gift of Dr Sankar Adhya. Poly(dl-dC)-(dl-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 mM concentration were incubated for 45 min with gel repressor at 20°C in 140 mM KCl, 25 mM Tris·HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT, 50 μM 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(dl-dC)-(dl-dC) and between $O_2$ and $O_3$ 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(dl-dC)-(dl-dC). Total operator DNA fragment concentration was 0.4 nM, with a 0.25 mM gel 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 $O_2$ in each component band and found not to affect the final results significantly.

Solute osmolarities were measured using a Wescor model 5100C vapor pressure osmometer. Even at the highest solute concentrations used for quantitating the osmotic effect, measured osmolarities 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 osmolar, respectively. Salt and solute osmolarities 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,

$$K_1 = \frac{F_{b1}(1-F_{b2})}{K_2(1-F_{b1})F_{b2}}$$

where $F_{b1}$ and $F_{b2}$ 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 $O_2$ and $O_3$ sites. For the competition between operator and poly(dl-dC)-(dl-dC), the fraction of non-specific sites with bound repressor can be estimated indirectly, analogous to (Fried, 1989). Assuming that the binding constant for 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(dl-dC)-(dl-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, $(F_{b0}-F_{b})_O C_O$, where $F_{b0}$ is the fraction operator with bound repressor either without added poly(dl-dC)-(dl-dC) or with a small reference concentration of poly(dl-dC)-(dl-dC) and $C_O$ is the concentration of operator sites. The expression for the ratio of operator and non-specific binding constants is then,

$$\frac{K_O}{K_{non}} = \frac{F_{b0}(1-F_{b})_O}{(1-F_{b0})(F_{b0}-F_b)_O} \frac{C_{non}}{C_O}$$

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

$$\left(1 - F_{b0}\right) \cdot \frac{F_{b0} - F_b}{F_{b0}} = \frac{K_{non}}{K_{non}} \cdot \frac{C_{non}}{C_O}$$

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