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LETTERS TO THE EDITOR

Equilibrium DNA-binding of AraC Protein

Compensation for Displaced Ions

Experiments on the AraC regulatory protein of *Escherichia coli* suggest a mechanism that DNA-binding proteins can use to reduce potentially drastic alterations in their affinity for DNA resulting from changes in salt concentration. Measurement of the net number of ions apparently displaced as AraC protein binds DNA and of fluorescence changes in the protein lead to the following picture. About 14 ions are displaced from the DNA as the protein binds the *ara*I site. As the protein binds the DNA, however, it undergoes a conformational change and binds about ten ions. Consequently, the net order of the reaction is reduced from 15th to about fourth order in salt concentration.

Since many DNA-binding proteins appear to contact closely ten to 20 phosphate groups on their DNA-binding sites, their binding likely involves the displacement of ten to 20 neutralizing charges either from the protein, or more likely, the DNA. Consequently, the affinity of such proteins for DNA could be extraordinarily sensitive to variations in intracellular salt concentration; $K_D \propto [\text{salt}]^{10-20}$. Since intracellular salt concentrations of *Escherichia coli* cannot be held constant (Schultz & Solomon, 1961; Epstein & Schultz, 1965; Munro *et al.*, 1972; Measures, 1975), the efficiency of gene regulation by DNA-binding proteins could be improved by a reduction in the net number of ions displaced in the overall binding reaction. This would reduce the amount that K_D varies with changing salt concentrations. It appears that the AraC regulatory protein of *E. coli* does precisely this. On one hand, AraC protein makes 15 ionic contacts with its binding site, *ara*I, since ethylation of any of 15 phosphate groups of the site interferes with binding by AraC protein (Hendrickson & Schleif, 1985). On the other hand, the variation in the affinity of the protein for the *ara*I site as a function of salt concentration (in the same range that the ethylation studies were done) indicates that the protein displaces a net of only four ions as it binds (Hendrickson & Schleif, 1984).

To examine the discrepancy between the number of physical phosphate contacts and the net number of ions displaced by AraC protein, we extended our measurements of binding affinity beyond the physiological salt concentration range used previously (Hendrickson & Schleif, 1984), utilized a number of different anions as well as Mg^{2+}, and also measured fluorescence of the protein both free and bound to DNA.

Measurements of binding affinity show that the variation of the dissociation constant with KCl concentration, $-\text{dlog}\,K_D/\text{dlog}\,[\text{KCl}]$, below 150 mM-KCl is about 4, as measured previously (Hendrickson & Schleif, 1984); however, above 200 mM-KCl it is about 14 (Fig. 1). Appropriate control experiments with *ara*I show that the binding is at equilibrium over the range of salt concentrations investigated, and is independent of DNA concentration and DNA length between 90 and 4000 base-pairs. Using Record and co-workers' analysis of ion effects (Record *et al.*, 1976; deHaseth *et al.*, 1977; Record *et al.*, 1978), these slopes indicate that four ions are displaced during the binding reaction at low salt and 14 to 16 ions are displaced at high concentrations of salt. The number of ions displaced at high concentrations of salt closely agrees with the 15 phosphate contacts observed in the *ara*I site (Hendrickson & Schleif, 1985). The binding data for the *ara*O_2 site (Dunn *et al.*, 1984) and non-specific DNA give similar results (Fig. 1). At low concentrations of salt, a net of only one ion is displaced as the protein binds to these other DNAs, while at high salt, 10 or 11 ions are displaced.

To test whether the unusual salt effects might be due to an effect of the ions on AraC protein itself and not on the complex DNA-binding reaction, we observed the effect of KCl concentration on the intrinsic fluorescence of the protein in solution. Figure 2 shows the fluorescence of AraC protein at an excitation wavelength of 280 nm, emission 337 nm, that likely is due primarily to the six tryptophan residues in the protein. In the absence of DNA, the observed fluorescence is approximately 20% greater at KCl concentrations above 100 mM than at KCl concentrations less than 50 mM. This increase in fluorescence likely reflects a conformational change in the protein that results from the interaction of AraC protein with salt ions.

Ions can alter the conformation of protein either through their effects on the solvent or by binding to the protein (Jencks, 1969; von Hippel & Schleich, 1969). If the effect of ions on a protein conformation is through the solvent, ordering various ions by the concentration at which they cause the alteration generally results in a lyotropic or Hofmeister series. The concentrations at which different monovalent ions interact with AraC protein does not follow this series, suggesting that a specific binding reaction may be occurring between AraC protein and salt ions. We observe the following

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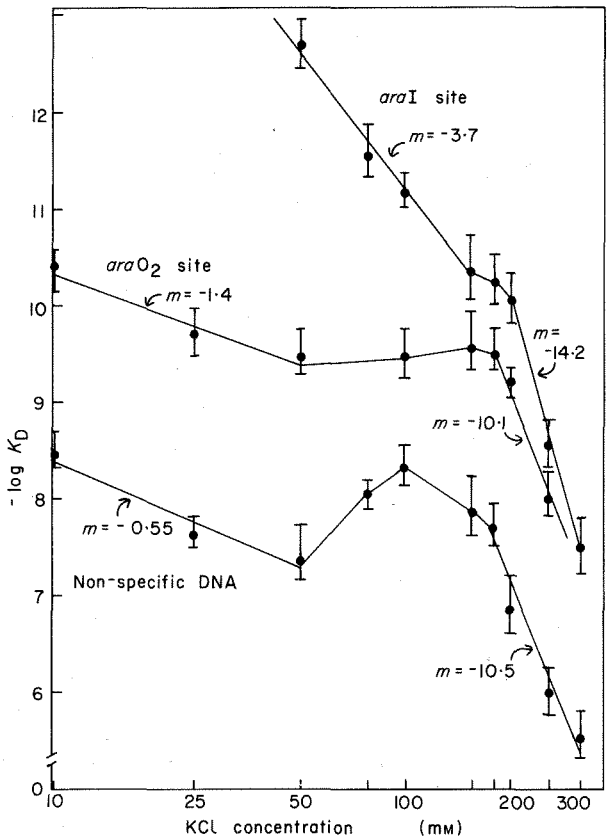

Figure 1. Log-log plot of AraC protein equilibrium dissociation constants at 35°C in the presence of the inducer arabinose (50 mM) for the *ara*I site, arabinose or the anti-inducer fucose (50 mM) for the *ara*O_2 site and non-specific DNA, as a function of KCl concentration. Error bars represent ±1 standard deviation of at least 3 measurements. Non-specific DNA constants are represented as per 30 base-pairs of DNA and were determined using various fragments of the ampicillin gene of the cloning vector pKO1 (McKenney *et al.*, 1981). The degree of homology between AraC binding sites and sequences on these fragments was evaluated by weighting each consensus position proportional to the number of times it occurs in the 6 AraC sites described (Hendrickson & Schleif, 1985) plus *ara*O_2, whose sequence is gAaaccaaTtgTCCATAttgcatCaG (upper case letters are consensus bases). A homology score was obtained by comparing each 26 or 27-base sequence (flexibility was allowed for the missing base near the left edge of the *ara*E site) possessing the 5 bases that are absolutely conserved in the AraC binding sites with the consensus sequence, summing the appropriately weighted values of the homologous bases, and dividing by the maximum possible score. The 7 AraC sites, *ara*I, *ara*E, *ara*O_1, *ara*H, *ara*FG_1, *ara*FG_2 and *ara*O_2, themselves score values of 96, 96, 79, 75, 76, 94 and 65%, respectively, whereas the 3 sites contained on the non-specific fragments score 57, 55 and 53%.

order of anions, listed in order of the concentration at which the salt-dependence of the *ara*O_2-binding reaction shifts from low to high:

$$Ac < NO_3 < I \approx PO_4 \approx Cl < SO_4 < \text{glutamate} \approx F.$$

A lyotropic series is:

$$I < NO_3 < Cl < Ac < PO_4 < SO_4 < F.$$

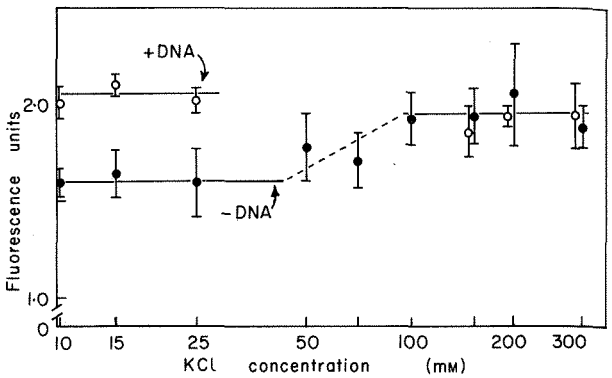

Figure 2. Fluorescence of AraC protein as a function of KCl concentration in the presence (○) and absence (●) of DNA containing the *ara*CBAD regulatory region on a 4000-base plasmid, pTD3 (Dunn & Schleif, 1984). Measurements were made on a Perkin-Elmer 650-40 fluorescence spectrophotometer; excitation wavelength, 280 nm; emission wavelength = emission maximum, 337 nm. The fluorescence cell and all buffer solutions were kept at 35°C. Immediately before use, purified AraC protein (Hendrickson & Schleif, 1984) was centrifuged at 50,000 revs/min at 4 to 10°C, for 1 h at 150 μg/ml in the buffer described below to remove any precipitate. For fluorescence measurements, AraC was diluted to 750 ng/ml, by adding buffer slowly to the protein to avoid extreme dilutions. Other dilution procedures result in strong fluorescence that does not change under different conditions. Many of the reported values were obtained blind with respect to salt concentration to avoid biases. Fluorescence measurements could be made up to 3 h after dilution of AraC for centrifugation. Values given are the uncorrected difference between the buffer with and without AraC protein and relative values were similar for the 4 AraC preparations tested. The buffer is nearly the same as that used for gel binding assays, containing 30 mM-arabinose, fucose or no sugar, 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA, 5% (v/v) glycerol, 10 to 300 mM-KCl, but with the omission of the detergent Nonidet-P40, and bovine serum albumin, which fluoresce, and reduction of dithioerythritol concentration to 1 μM, which absorbs near the exciting wavelength when oxidized. Error bars represent ±1 standard deviation of at least 5 measurements when no DNA was present, at least 2 measurements in the presence of DNA.

In light of the salt-induced variation in K_D and the fluorescence results, we suggest the following model. At relatively low concentrations of salt, binding to the *ara*I site by the protein is accompanied by the displacement of around 14 ions from the DNA and the binding of about ten ions by the protein, leaving the net order of the salt-dependence of the reaction as four. At high concentrations of salt, the 14 ions are still displaced as the protein binds, but the protein is already saturated with ions and unable to bind more. The order of this reaction with respect to salt is about 14.

The model described above, which explains how AraC protein can compensate for salt effects from displaced ions, is supported by the fluorescence of AraC protein bound to DNA. Since the model proposes that AraC protein binds salt when it binds

DNA at low concentrations of salt, and since the apparent salt-binding reaction involves a conformational change and fluorescence increase by AraC protein, a similar conformational change and fluorescence increase should occur when AraC binds to DNA at low concentrations of salt. This prediction is met. The additional data in Figure 2 show that, as predicted, the fluorescence of AraC protein bound to DNA is about 25% greater than that of free protein.

Since Mg^{2+} is generally assumed to be present in the cell at effective concentrations of 2 to 3 mM, and is known to have a large effect on the affinity of DNA-binding proteins (Record *et al.*, 1977) including AraC protein (Hendrickson & Schleif, 1984), we have observed its effect on the ion compensation mechanism. Variation in the concentration of $MgCl_2$ alone, from 0·17 to 15 mM, results in a slope, dlog K_D/dlog $[Mg^{2+}]$, of $-1{\cdot}6$ using the *ara*I site. This is about half of the "low salt" slope observed with a monovalent ion, dlog K_D/dlog $[K^+] = -3{\cdot}6$. As concluded by Hendrickson & Schleif (1984), this indicates that cations are the predominant determinant of the ionic dependence of the DNA-binding reaction of AraC protein (Kowalczykowski *et al.*, 1981), and also that the proposed ion compensation mechanism functions for Mg^{2+}, perhaps even more effectively than for monovalent ions.

We have also observed the effect of physiological Mg^{2+} concentration on the KCl-dependent affinity of AraC for *ara*I. The presence of 2·5 mM-Mg^{2+} in the binding reaction virtually eliminates the variation in affinity for *ara*I as $[K^+]$ is varied over the range of 10 to 200 mM, while at KCl concentrations greater than 200 mM, the DNA-binding affinity drops off sharply (data not shown). This can be understood, since Mg^{2+} will act as a simple competitor with K^+ counterions on the DNA, thus reducing the number of K^+ ions displaced as the protein binds. Such an effect of Mg^{2+} in reducing the variation in K_D with KCl concentration has been observed for the binding of *lac* repressor and RNA polymerase to DNA (Record *et al.*, 1976, 1977; Shaner *et al.*, 1982. At KCl concentrations greater than 200 mM, however, the extent to which 2·5 mM-Mg^{2+} can compete is insignificant and the steep slope, also observed in the absence of Mg^{2+} (Fig. 1), results.

Data compatible with the mechanism we propose have been observed in several other systems. Terry *et al.* (1983) observed an increase in the dependence of the equilibrium binding reaction on salt concentration when NaCl concentration was raised above 100 mM for the *Eco*RI endonuclease, as did Kowalczykowski *et al.* (1981) for the phage T4 gene *32* protein. The latter group presented evidence that at low concentrations of salt, a conformational change occurs in gene *32* protein, resulting in a non-binding form of the protein. Also, for *lac* repressor, the dependence of the DNA-binding constants, K_A and k_A, on salt concentration increases at high concentrations of salt (Winter *et al.*, 1981; Barkley, 1981). Barkley (1981) has discussed a model similar to that presented for AraC protein to explain this; however, in the absence of evidence that the *lac* repressor binds salt ions or changes conformation in the relevant salt range, she favored a model where association rates are reduced at low concentrations of salt due to decreased sliding of the protein along the DNA. This explanation is not applicable to the equilibrium data that have been presented for AraC protein. Further, we observe high and low salt slopes, dlog K_D/dlog [KCl], identical to those presented for *ara*O_2 for a situation that should eliminate virtually all effects due to adjacent, non-specific DNA: a 33 base-pair synthetic *ara*I site (data not shown).

In conclusion, the salt-dependence of AraC protein's affinity for the *ara*I site, within physiological salt concentration ranges, is considerably less than would be expected from the number of DNA phosphate residues the protein appears to contact. This decreased salt-dependence would reduce alterations in gene regulation due to changes in intracellular salt concentration that occur in response to changes in external osmolarity, age of the culture, pH of the medium, and energy source (Schultz & Solomon, 1961; Epstein & Schultz, 1965). (Since ion concentrations can change by at least 6-fold (Epstein & Schultz; 1965), if a net of 15 ions were displaced as the protein bound, the affinity of the protein could vary by a factor of over 10^7.) The decreased sensitivity to salt of AraC protein's DNA-affinity appears to result from the binding of about ten ions to the protein as it undergoes a conformational change while binding to DNA. This interpretation is consistent with the salt-dependent affinity of the protein for variant binding sites, the fact that the protein's affinity for DNA shows a very high dependence on salt concentration at high concentrations, and the salt and DNA-dependent fluorescence changes of the protein. Other DNA-binding proteins must also regulate genes independently of salt concentration and may use similar mechanisms of ion compensation.

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