The Salt Dependence of the Interferon Regulatory Factor 1 DNA Binding Domain Binding to DNA Reveals Ions Are Localized around Protein and DNA

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ABSTRACT: The equilibrium dissociation constant of the DNA binding domain of interferon regulatory factor 1 (IRF1 DBD) for its DNA binding site depends strongly on salt concentration and salt type. These dependencies are consistent with IRF1 DBD binding to DNA, resulting in the release of cations from the DNA and both release of anions from the protein and uptake of a cation by the protein. We demonstrated this by utilizing the fact that the release of fluoride from protein upon complex formation does not contribute to the salt concentration dependence of binding and by studying mutants in which charged residues in IRF1 DBD that form salt bridges with DNA phosphates are changed to alanine. The salt concentration dependencies of the dissociation constants of wild-type IRF1 DBD and the mutants R64A, D73A, K75A, and D73A/K75A were measured in buffer containing NaF, NaCl, or NaBr. The salt concentration and type dependencies of the mutants relative to wild-type IRF1 DBD provide evidence of charge neutralization by solution ions for R64 and by a salt bridge between D73 and K75 in buffer containing chloride or bromide salts. These data also allowed us to determine the number, type, and localization of condensed ions around both IRF1 DBD and its DNA binding site.

Counterions are condensed on DNA and reduce its charge density (*1–9*). These counterions are released from the DNA into the solution when charged protein residues form salt bridges with DNA phosphates. The release gives rise to the salt concentration dependence of binding, which has been widely interpreted to be proportional to the net number of ions released from the DNA (*10–14*).

It has been assumed (*15–19*) that because proteins have a low charge density compared to DNA, counterions will not condense on charged residues of proteins, and thus, they will not contribute to the salt concentration dependence of binding. In many DNA binding proteins, however, basic residues are clustered at the DNA binding interface, which generates a high local positive charge density. Therefore, it seems possible that condensation of counterions on the DNA binding surface of proteins, which would generate a salt concentration dependence of binding, may occur. In fact, evidence suggesting counterions are concentrated in excess of co-ions around some proteins exists, and the release of these condensed counterions from the protein upon DNA binding contributes to the salt concentration dependence of binding. This evidence includes sensitivity of the equilibrium binding constant to anion identity (*20–22*) and nonlinearity over large ranges of salt concentrations of log–log plots of the binding constant versus salt concentration (*22–25*). In contrast to local ion effects, redistribution of global ions around the protein and DNA has been proposed as a source of the salt concentration dependence of binding (*26*).

Here we describe the effects of ions on DNA binding for the interferon regulatory factor 1 DNA binding domain, IRF1 DBD.[1] Charged residues, whose positions in the protein−DNA complex allow them to form salt bridges with DNA phosphates, were mutated to alanine, and the salt concentration and type dependencies of binding of the mutant proteins were measured. The salt concentration dependencies of the K_d values of the mutants relative to wild-type IRF1 DBD revealed whether these charged residues are primarily neutralized by solution ions or primarily form a salt bridge with a neighboring residue with the opposite charge. Using this information and the fact that the release of fluoride ions from protein does not contribute to the salt concentration dependence of binding (*27*), we demonstrated that ions are condensed on IRF1 DBD and its DNA binding site, and we determined the number, type, and average degree of condensation of ions around DNA and three charged residues in IRF1 DBD.

MATERIALS AND METHODS

Purification of IRF1 DBD. A pET-3a plasmid (Novagen) encoding the 113 N-terminal amino acids comprising the DNA binding domain of interferon regulatory factor 1 was provided by P. Privalov (*28*). The expression and purification of IRF1 DBD have been adapted from Escalante et al. (*29*). A single colony of cells, BL21(DE3), freshly transformed with pET3a-IRF1 DBD, was grown overnight at 37 °C in 100 mL of YT medium (*30*) containing 100 μg/mL ampicillin. The next morning the cells were washed and resus-

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[1] Abbreviations: IRF1 DBD, interferon regulatory factor 1 DNA binding domain; FAM, 6-carboxyfluorescein; PRDI, positive regulatory domain I.

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pended in 100 mL of fresh YT medium and added to two 2 L baffled flasks, each containing 500 mL of YT medium supplemented with 100 μg/mL ampicillin. The cells were grown at 37 °C to an apparent OD_{600} of ~0.6−0.8. At this point, IPTG was added to a final concentration of 0.4 mM and growth was continued for 4 h, after which the cells were pelleted and frozen.

The frozen cells were resuspended in 3 volumes of lysis buffer [50 mM HEPES (pH 7.5), 0.5 M KCl, 10% glycerol, 0.1% Triton X-100, 1 mM EDTA, 2 mM DTE, and 0.2 mM PMSF] and lysed by being passed three times through a French press. DNase I (10 μg/mol) was added after lysis. The insoluble fraction, which contains IRF1 DBD, was pelleted and solubilized in 25 mL of lysis buffer containing 6 M guanidine hydrochloride. The 25 mL IRF1 DBD sample was refolded by dialyzing it against small volumes of lysis buffer such that when equilibrium was attained, the internal and external concentrations of guanidine hydrochloride were 2, 1, 0.5, or 0.25 M. The sample after each dialysis step was allowed to equilibrate for 3 h at 4 °C. The final dialysis was against 10 mM sodium phosphate (pH 7.4), 0.3 M NaCl, 5% glycerol, and 2 mM DTE. This 25–30 mL protein solution, in which IRF1 DBD was at a concentration of approximately 75 μM and 90% pure, was loaded onto a 5 mL HiTrap Heparin HP column (Pharmacia) pre-equilibrated with the final dialysis buffer. IRF1 DBD eluted at 640 mM NaCl in a 60 mL gradient from 300 to 800 mM NaCl. Heavily overloaded samples run on SDS−14% (w/v) polyacrylamide gels showed no trace of contaminating proteins. Pure IRF1 DBD was stored at –70 °C. Concentrations of IRF1 DBD were determined using an extinction coefficient (ϵ_{280}) of 36820 M^{-1} cm^{-1} as calculated from the amino acid composition by the method of Pace et al. (*31*). Ten grams of cells typically yielded 20–40 mg of pure IRF1 DBD.

The mutations R64A, D73A, K75A, and D73A/K75A were introduced into the pET3a-IRF1 DBD plasmid using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Plasmid DNA was isolated from cells using the Wizard Plus Miniprep DNA purification system from Promega (Madison, WI). The mutations were confirmed by DNA sequencing, and the mutant proteins were expressed and purified as described for wild-type (WT) IRF1 DBD. While WT IRF1 DBD and the R64A mutant protein eluted at 640 mM NaCl, the K75A and D73A/K75A mutant proteins eluted at 555 mM NaCl, and the D73A mutant protein eluted at 725 mM NaCl. The proteins eluted at high concentrations from the column, so salt carried over to the experimental buffer was insignificant.

Fluorescence Anisotropy Measurement of DNA Binding. A 13 bp oligonucleotide with the sequence 5′-AC<u>TTTCACT</u>-<u>T</u>CTC-3′ labeled at the 5′ end with the fluorescent tag 6-carboxyfluorescein (FAM) and its unlabeled complement were purchased from Integrated DNA Technologies, Inc. The positive regulatory domain I (PRD I) site, the binding site of IRF1 DBD, from the human interferon-β enhancer is underlined. We prepared the DNA duplex by mixing the complementary oligonucleotides in equimolar amounts in 10 mM sodium phosphate (pH 7.4) and 0.2 M NaCl, heating the mixture to 70 °C, and cooling it overnight.

To assess binding, increasing amounts of pure IRF1 DBD were mixed in a cuvette containing 2.4 mL of 5×10^{-9} M DNA in 10 mM sodium phosphate (pH 7.4), 2 mM DTE, and either NaF, KF, NaCl, KCl, NaBr, or KBr between 200 and 650 mM. Measurements were taken on a T-format Photon Technology International (Lawrenceville, NJ) spectrofluorimeter with a 75 W xenon arc lamp. The FAM label was excited at 490 nm, and its emission at 520 nm was measured. The fluorescence anisotropy was calculated from the measured fluorescence intensities, which were corrected for the *G* factor. The K_d of the IRF1 DBD−DNA binding reaction was determined by least-squares fitting the curve of fluorescence anisotropy as a function of total protein concentration to the following binding equation:

$$A = A_0 + \Delta A \frac{P_t + D_t + K_d - \sqrt{(P_t + D_t + K_d)^2 - 4P_tD_t}}{2D_t}$$

where A_0 is the anisotropy of free DNA, ΔA is the change in anisotropy between the maximum anisotropy of the complex and the anisotropy of free DNA, P_t is the total protein concentration, D_t is the total DNA concentration, and K_d is the protein−DNA dissociation constant.

Measuring Slope and Standard Error. The log of the K_d was plotted versus the log of the salt concentration, and the slope of this function was calculated for WT IRF1 DBD in six salts and for each of the four mutants in three salts. The standard error of the data points with respect to their least-squares fit was calculated using the LINEST statistical function in Microsoft Excel.

Fluorescence Intensity of Guanidine Hydrochloride-Induced Unfolding. Increasing amounts of guanidine hydrochloride were mixed in a cuvette containing 2.4 mL of 1×10^{-6} M IRF1 DBD or one of the four mutants in 10 mM sodium phosphate (pH 7.4), 0.3 M NaCl, and 2 mM DTE. The protein was excited at 280 nm, and the emission at 336 nm was measured. Denaturation curves of the intrinsic fluorescence normalized to that of native protein versus guanidine hydrochloride concentration were plotted and compared for WT IRF1 DBD and the four mutant proteins.

RESULTS

Salt Concentration and Type Dependencies of the K_d for Interaction of IRF1 DBD with Its DNA Binding Site. To determine the ionic contributions of IRF1 DBD binding to its DNA binding site, we monitored the equilibrium binding of the protein to a 13 bp FAM-labeled DNA duplex by fluorescence anisotropy. The intensity of the fluorescence anisotropy signal increased with an increase in IRF1 DBD concentration as a result of decreasing the tumbling rate of the DNA upon formation of the protein−DNA complex and fixing the fluorescent probe on the protein. From this information, we calculated the equilibrium dissociation constant of IRF1 DBD for DNA. A typical binding curve for WT IRF1 DBD in 300 mM NaCl is shown in Figure 1.

The association of protein, P, and DNA, D, to form the protein−DNA complex, PD, in buffer containing salt, MX, may be represented by

$$P \cdot nX^- + D \cdot qM^+ + mM^+ \Leftrightarrow PD \cdot mM^+ + nX^- + qM^+ \quad (1)$$

where *m* represents the number of cations taken up by the protein, *n* represents the number of anions released from the

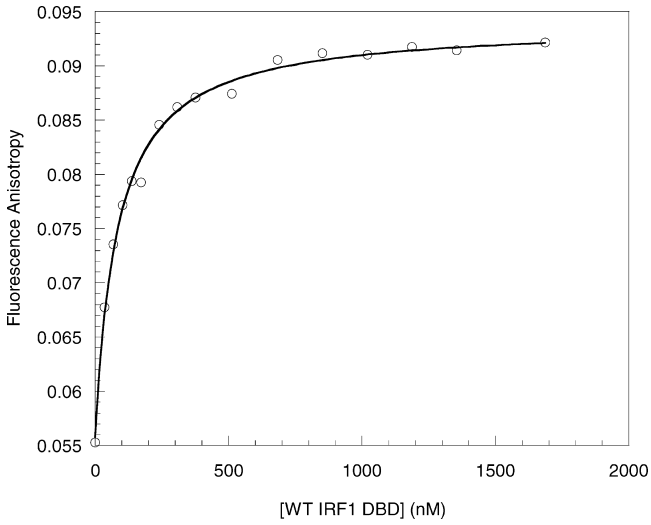

FIGURE 1: Representative binding curve of WT IRF1 DBD binding to a FAM-labeled 13 bp oligonucleotide at 300 mM NaCl. The fluorescence anisotropy is shown as a function of protein concentration. The fluorescent label was excited at 490 nm, and its emission was measured at 520 nm. The solid curve is the nonlinear least-squares fit of the binding equation to the data.

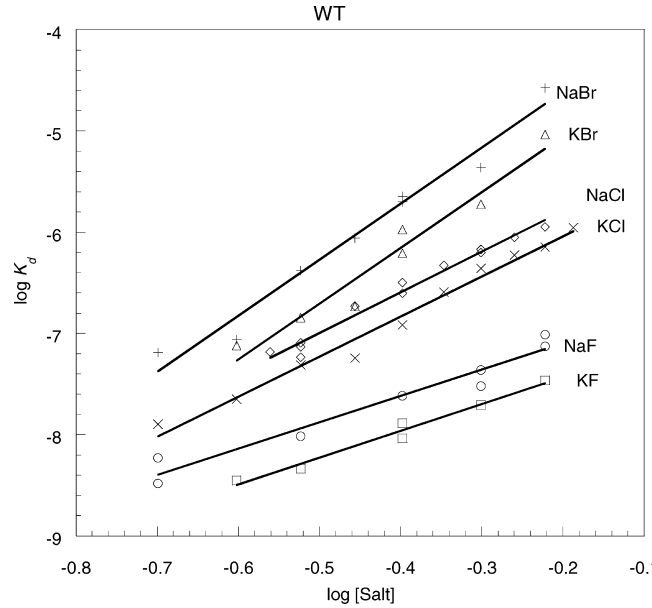

FIGURE 2: Dependence of log K_d of WT IRF1 DBD on the logarithm of the NaF, KF, NaCl, KCl, NaBr, and KBr concentrations. The K_d values were obtained from individual binding experiments.

protein, and q represents the number of cations released from the DNA upon complex formation (*24*).

The following equation for the salt concentration dependence of binding can be derived from the power law dependence inherent in eq 1 by analogy to Record et al. (*10*) and Record et al. (*32*):

$$\partial \log K_d / \partial \log [MX] = q - m + n \quad (2)$$

Equation 2 relates the slope of the plot of log K_d versus the log of the salt concentration to the effective net number of ions released. Each of the variables in eq 2 can be partitioned into the number of ion binding sites and the thermodynamic association of ions per site for DNA and protein as shown in the following equation:

$$\partial \log K_d / \partial \log [MX] = b\psi - c\varphi + a\delta \quad (3)$$

In eq 3, b is the number of DNA phosphates that form salt bridges with protein residues and ψ is the average degree of condensation or average effective concentration of cations per DNA phosphate. The number of cations released from the DNA (q) is the product of b and ψ. The number of anionic residues of IRF1 DBD that take up cations upon DNA binding is c, and the average degree of condensation of cations per site is φ. The number of cations taken up by the protein (m) is the product of c and φ. The number of cationic residues that release anions upon DNA binding is a, and the average degree of condensation of anions per site is δ. Different anions are likely to condense to different degrees due to their different sizes, so we assign fluoride δ_1, chloride δ_2, and bromide δ_3. The number of anions released from the protein (n) is the product of a and δ. If counterions are completely condensed on DNA and protein, ψ, φ, and δ will equal 1. If counterions are uncondensed, ψ, φ, and δ will equal 0. In this paper, we determine ψ, φ, δ_2, and δ_3 for three charged residues in IRF1 DBD and its DNA binding site.

Over the range of concentrations used (200−650 mM) of the six salts that were studied (NaF, KF, NaCl, KCl, NaBr, and KBr), the plot of log K_d versus the log of the salt concentration is linear as shown in Figure 2. Each data point in Figure 2 is derived from one binding curve, an example of which is shown in Figure 1. Aggregation of IRF1 DBD at salt concentrations below 200 mM prevented us from extending the range of salt concentration dependence studies below 200 mM. The increase in K_d with an increase in salt concentration between 200 and 650 mM indicates a net release of ions, cations and/or anions, from the protein and/or DNA upon complex formation. We measured the K_d in a variety of salts to extract the relative contributions of cation and anion release to the slopes of the plots in Figure 2. When we state that an experiment was performed in buffer containing a certain salt, that is the only salt the experimental buffer contained. Table 1 shows the salt concentration dependencies of WT IRF1 DBD binding to DNA in buffers containing these salts.

Table 1: Salt Dependence of Binding of WT and Mutant IRF1 DBD

protein	salt	slope	standard error
WT	NaF	2.60	0.23
	KF	2.65	0.20
	NaCl	4.02	0.19
	KCl	3.95	0.19
	NaBr	5.53	0.38
	KBr	5.51	0.57
R64A	NaF	1.31	0.14
	NaCl	2.37	0.10
	NaBr	3.36	0.36
D73A	NaF	2.95	0.32
	NaCl	6.21	0.51
	NaBr	8.12	0.41
K75A	NaF	1.55	0.11
	NaCl	4.20	0.16
	NaBr	5.16	0.29
D73A/K75A	NaF	1.17	0.20
	NaCl	4.29	0.13
	NaBr	5.13	0.22

Cation identity affects the K_d of IRF1 DBD for DNA, but it does not affect the salt concentration dependence of the K_d. That is, binding of IRF1 DBD to DNA is tighter in potassium salts than in sodium salts with the same anion, but the salt concentration dependencies of the K_d values do

Table 2: Net Numbers of Ions Released by Different Structural Elements when IRF1 DBD Binds DNA

				potential no. of ions released from element		
case	element	status when free	status in complex	M^{+a}	F^b	Cl^- or Br^{-c}
a	DNA phosphate	neutralized by solution ions	forms a salt bridge with a basic residue	+1	0	0
b	basic residue	neutralized by solution ions	forms a salt bridge with a DNA phosphate	0	0	+1
c	acidic residue	neutralized by solution ions	neutralized by solution ions	0	0	0
d	basic residue	neutralized by a salt bridge with an acidic residue	forms a salt bridge with a DNA phosphate	0	0	0
e	acidic residue	neutralized by a salt bridge with a basic residue	neutralized by solution ions	−1	0	0
f	neutral residue	−	−	0	0	0

[a] Where M^+ is Na^+ or K^+. [b] If the buffer anion is Fl^-, this column applies. [c] If the buffer anion is Cl^- or Br^-, this column applies.

not differ significantly from one another (Figure 2 and Table 1). The slopes are approximately 2.6 in potassium and sodium fluoride, 4.0 in potassium and sodium chloride, and 5.5 in potassium and sodium bromide (Table 1). These similar slopes indicate that similar net numbers of potassium and sodium ions are released upon complex formation.

In contrast to cation type, anion type affects both the magnitude of the K_d and the salt concentration dependence of the K_d (Figure 2 and Table 1). That is, when the anion type is changed and the cation type is kept constant, the dissociation constants differ significantly (Figure 2). Binding is tightest in fluoride salts, intermediate in chloride salts, and weakest in bromide salts. The salt concentration dependence of the K_d is also sensitive to anion type. The slope of this function is 2.60, 4.02, and 5.53 in buffer containing sodium fluoride, sodium chloride, and sodium bromide, respectively (Table 1). The different slopes demonstrate that different numbers of fluoride, chloride, and bromide ions are displaced from IRF1 DBD upon binding DNA. The fact that the release of chloride and bromide ions from IRF1 DBD contributes to the salt concentration dependence of binding demonstrates that these anions are condensed on free IRF1 DBD.

R64 Releases an Anion upon IRF1 DBD−DNA Binding. To learn more about charge neutralization in IRF1 DBD, we examined the ionic contributions from individual basic residues. In the protein−DNA complex, arginine 64 forms a salt bridge with a DNA phosphate. The ϵ-nitrogen of arginine 64 is 3.1 Å from the nearest phosphate oxygen of thymine 215 (PDB entry 1IF1). In free IRF1 DBD, solution ions must neutralize the positive charge of R64 because the protein does not contain a nearby negative residue that could neutralize R64 by forming a salt bridge. To verify that solution ions neutralize the positive charge of R64, we compared the experimentally measured differences between the salt concentration dependencies of binding for the R64A mutant and WT IRF1 DBD to the predicted number of ions that would be released if residue R64 were neutralized by solution ions. First, we demonstrated the expected net number of ions are released under conditions in which there was no contribution from interaction of anion with the protein. Then we used conditions under which release of anion from the protein contributed to the salt concentration dependence. Thus, any observed differences must be attributed to condensed anions interacting with the protein, specifically solution anions neutralizing R64.

To simplify the discussion here and in later sections, we partition the release and uptake of ion from a residue of IRF1 DBD and a DNA phosphate into the sum of one or more of the five partial reactions shown in Table 2. In this table, we represent the number of ions released as positive values and the number of ions taken up as negative values. Because of the large energy of dehydration, fluoride ions interact weakly with proteins. Larger ions, like chloride and bromide, can more easily become dehydrated, so they interact with proteins more strongly (*33*). Thus, we provide one column for fluoride and one for chloride and bromide.

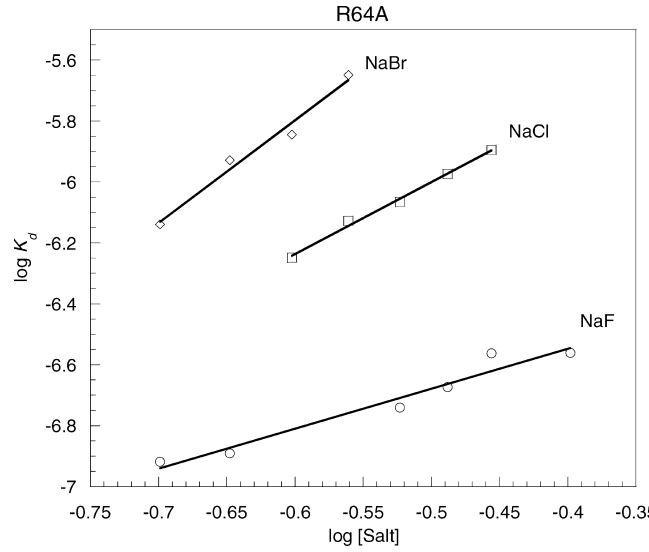

FIGURE 3: Dependence of log K_d of the R64A mutant protein on the log of the NaF, NaCl, or NaBr concentration. The K_d values were obtained from individual binding experiments.

Consider first the case of fluoride ions. The potential net release of ions upon binding of IRF1 DBD to DNA with respect to residue R64 is described by the sum of cases a and b, which is one ion, in buffer containing NaF (Table 2). When the mutant protein containing the R64A substitution binds DNA, case f describes the situation, and no ions are released with respect to this residue. The result in buffer containing NaF would therefore be a salt concentration dependence of binding approximately 1 unit smaller for the R64A mutant than for WT. As expected, experimentally we find the salt concentration dependence of binding is 2.60 ± 0.23 for WT IRF1 DBD and is 1.31 ± 0.14 for R64A in buffer containing NaF (Figure 3 and Table 1). The difference of approximately 1 in the slopes of the WT protein and the mutant protein must arise from the R64A mutant displacing one less cation from the DNA phosphates compared to WT

Table 3: Potential Contributions of Ions to the Salt Concentration Dependence of DNA Binding Attributable to Residues 75 and 73

free protein	partial reactions from Table 2	net no. of ions released in Fl^- buffer[a]	net no. of ions released in Cl^- or Br^- buffer[a]
WT, neutralization of K75 and D73 by solution ions	a + b + c	1	2
WT, neutralization of K75 and D73 by salt bridge	a + d + e	0	0
K75A	c + f	0	0
D73A	a + b + f	1	2
K75A/D73A	f	0	0

[a] As calculated from the entries in Table 2.

IRF1 DBD. This difference is also consistent with residue R64 being neutralized by a fluoride ion in the free protein.

Now we consider the cases of chloride and bromide ions. Chloride and bromide ions interact more strongly with protein than fluoride ions interact with protein (*33*), and the release of these ions from proteins upon DNA binding contributes to the salt concentration dependence of binding (*20, 21, 34*). If residue R64 of WT IRF1 DBD is neutralized by chloride, then when the protein binds DNA, the net release of ions with respect to R64 is described by the sum of cases a and b in the M^+ and Cl^- columns of Table 2, or a net of two ions. The R64A mutant protein's net ion release with respect to residue R64 would correspond to case f in Table 2, which is zero. Experimentally, we found this to be true; the salt concentration dependence of the K_d is smaller for R64A (2.37 ± 0.10) than for WT IRF1 DBD (4.02 ± 0.19) in buffer containing NaCl (Figure 3 and Table 1). The difference, 1.6, indicates not only that a sodium ion is condensed on the DNA phosphate, which forms a salt bridge with R64 in the complex, but also that a chloride ion must be condensed on residue R64 in free IRF1 DBD. Similar results were observed in buffer containing NaBr. The salt concentration dependence of the K_d is 5.53 ± 0.38 for WT IRF1 DBD and 3.36 ± 0.36 for the R64A mutant in buffer containing NaBr. Therefore, the positive charge of R64 in IRF1 DBD is neutralized by a solution ion in buffer containing NaCl or NaBr.

K75 and D73 Are Neutralized by Ions in Fluoride-Containing Buffer but Not in Chloride- or Bromide-Containing Buffers. In the IRF1 DBD−DNA complex, lysine 75 forms a salt bridge with a DNA phosphate. The ϵ-amino group of lysine 75 is 2.7 Å from the nearest phosphate oxygen of adenine 204. Unlike the environment of R64, however, a negatively charged residue, D73, lies near K75. Although a dehydrated salt bridge is usually 3 Å in length (*35*), the charged groups of amino acids D73 and K75 are 7 Å apart in the cocrystal structure of IRF1 DBD with DNA (*36*). It is possible that in the absence of DNA, D73 and K75 possess substantial salt bridge character. To determine how K75 is neutralized, we mutated this residue to an alanine and again measured the salt concentration and type dependencies of binding. We also made the single mutant D73A and the double mutant D73A/K75A to further test the hypothesis that D73 and K75 form a salt bridge in the free protein.

Table 3 presents the five situations being considered here and their breakdown into the partial reactions from Table 2,

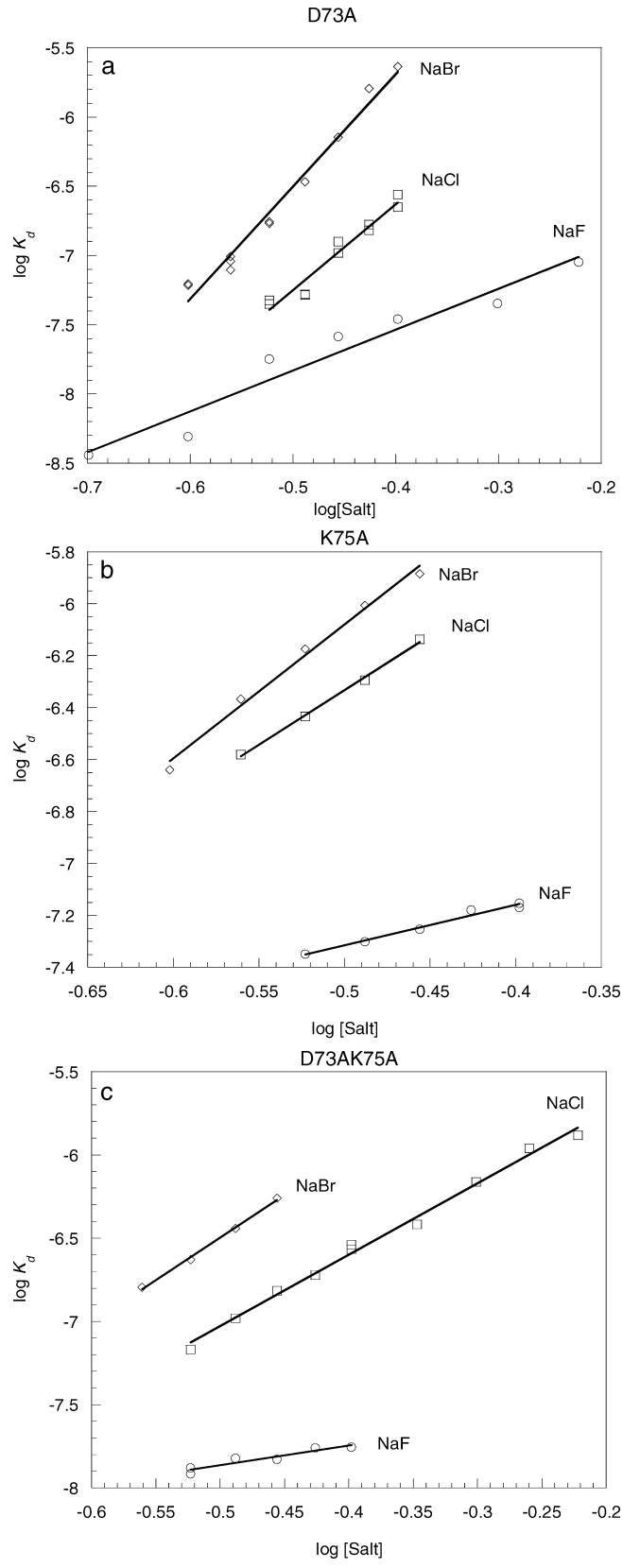

FIGURE 4: Dependencies of log K_d of the D73A (a), K75A (b), and D73A/K75A (c) mutant proteins on the logarithm of the NaF, NaCl, and NaBr concentrations. The K_d values were obtained from individual binding experiments.

with the resulting prediction of the net ion release for each of the cases in both fluoride buffer and chloride or bromide buffer. Experimentally, the data in fluoride buffers indicate D73 and K75 are neutralized by solution ions. The depen-

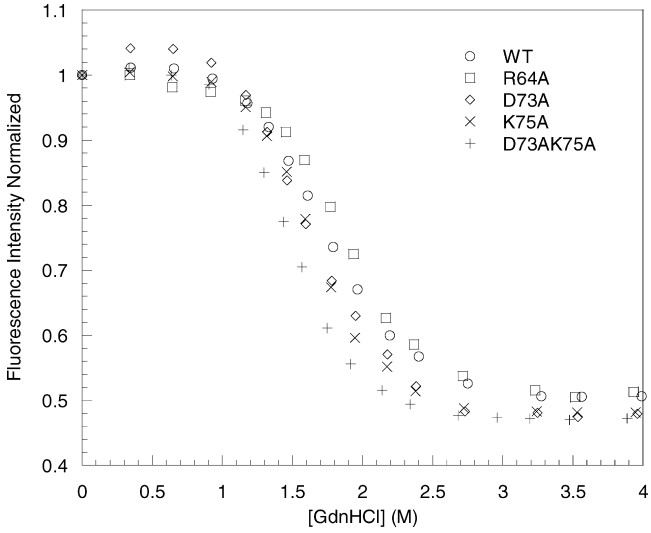

FIGURE 5: Dependence of the intrinsic fluorescence intensity, normalized to that of the native protein, on the concentration of GdnHCl for WT IRF1 DBD and the mutant proteins R64A, D73A, K75A, and D73A/K75A. The excitation wavelength was 280 nm, and the emission at 336 nm was measured.

dence of K_d on salt concentration in NaF for the D73A mutant is 2.95 ± 0.23 (Figure 4a), which is approximately equal to that of WT, 2.60 ± 0.23 (Figure 2), as seen in Table 1. The slopes of these plots for the K75A (Figure 4b) and D73A K75A (Figure 4c) mutants in NaF (1.55 ± 0.11 and 1.17 ± 0.20, respectively) were similar to each other and smaller than those of WT and the D73A mutant, demonstrating that residue K75 is neutralized by a solution ion in buffer containing NaF.

Experimentally, the salt bridge case was observed for residues D73 and K75 in buffer containing chloride or bromide salts. In the presence of NaCl, the slopes are 4.02 ± 0.19 for WT IRF1 DBD (Figure 2), 4.20 ± 0.16 for the K75A mutant (Figure 4b), and 4.29 ± 0.13 for the D73A/K75A mutant (Figure 4c). These slopes are approximately equal and smaller than the slope of the D73A mutant, 6.21 ± 0.51 (Figure 4a and Table 1). These results demonstrate that residue K75 forms a salt bridge with residue D73 in buffer containing chloride salt (Table 3). Similar data were collected in bromide salt. In the presence of NaBr, the slopes are 5.53 ± 0.38 for WT IRF1 DBD (Figure 2), 5.16 ± 0.29 for the K75A mutant (Figure 4b), and 5.13 ± 0.22 for the D73A/K75A mutant (Figure 4c). These slopes are approximately equal to each other and smaller than the slope of the D73A mutant, 8.12 ± 0.41 (Figure 4a and Tables 1 and 3). Therefore, residues D73 and K75 form a salt bridge in chloride and bromide salts.

The Mutant Proteins Are Folded. To ensure the mutations do not destabilize the proteins, which could potentially create additional sources of ion effects, we measured the fluorescence intensity upon guanidine hydrochloride-induced unfolding (Figure 5). The proteins unfolded in a similar fashion, and their calculated Gibbs free energies of unfolding are similar (data not shown). Additional evidence that the mutant proteins are folded in a conformation similar to that of WT IRF1 DBD is their ability to bind DNA.

Global Analysis of Ion Effects. The experiments and analysis described above have shown that for residues 64, 73, and 75, the release of ions from IRF1 DBD and DNA and the uptake of ions by IRF1 DBD contribute to the salt concentration dependence of the binding of the protein to DNA. The fact that such effects were observed requires that the degree of condensation be appreciable. In this section, we will estimate the magnitude of the condensation by a global analysis of the binding data.

By analogy to eq 3, the following system of equations describes the salt concentration dependence of binding for WT IRF1 DBD and the mutant proteins R64A, D73A, K75A, and D73A/K75A in the presence of NaF, NaCl, or NaBr. The analysis is global because the left-hand sides of the equations express the slopes in terms of the residue, phosphate, mode of neutralization, and degree of condensation. The right-hand sides of the equations are the experimentally determined values of the salt concentration dependence of binding taken from Table 1.

$$\text{WTNaF:}\quad b\psi - (c-1)\varphi + a\delta_1 = 2.60 \pm 0.23 \tag{4}$$

$$\text{WTNaCl:}\quad b\psi - c\varphi + (a-1)\delta_2 = 4.02 \pm 0.20 \tag{5}$$

$$\text{WTNaBr:}\quad b\psi - c\varphi + (a-1)\delta_3 = 5.53 \pm 0.38 \tag{6}$$

$$\text{R64ANaF:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_1 = 1.31 \pm 0.14 \tag{7}$$

$$\text{R64ANaCl:}\quad (b-1)\psi - c\varphi + (a-2)\delta_2 = 2.37 \pm 0.10 \tag{8}$$

$$\text{R64ANaBr:}\quad (b-1)\psi - c\varphi + (a-2)\delta_3 = 3.36 \pm 0.36 \tag{9}$$

$$\text{D73ANaF:}\quad b\psi - (c-1)\varphi + a\delta_1 = 2.95 \pm 0.32 \tag{10}$$

$$\text{D73ANaCl:}\quad b\psi - (c-1)\varphi + a\delta_2 = 6.21 \pm 0.51 \tag{11}$$

$$\text{D73ANaBr:}\quad b\psi - (c-1)\varphi + a\delta_3 = 8.12 \pm 0.41 \tag{12}$$

$$\text{K75ANaF:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_1 = 1.55 \pm 0.11 \tag{13}$$

$$\text{K75ANaCl:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_2 = 4.20 \pm 0.16 \tag{14}$$

$$\text{K75ANaBr:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_3 = 5.16 \pm 0.29 \tag{15}$$

$$\text{D73A/K75ANaF:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_1 = 1.17 \pm 0.20 \tag{16}$$

$$\text{D73A/K75ANaCl:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_2 = 4.29 \pm 0.13 \tag{17}$$

$$\text{D73A/K75ANaBr:}\quad (b-1)\psi - (c-1)\varphi + (a-1)\delta_3 = 5.13 \pm 0.22 \tag{18}$$

Using information from the cocrystal structure of the IRF1 DBD−DNA complex (*36*), we solved the system of equations for the following unknowns: ψ, average degree of condensation of cations per DNA phosphate; φ, average degree of condensation of cations per acidic residue, which, in the free protein, forms a salt bridge with a basic residue that forms a salt bridge with a DNA phosphate in the complex; and δ_2 and δ_3, average degrees of condensation of chloride and bromide ions, respectively, per basic residue of IRF1 DBD that forms a salt bridge with a DNA phosphate.

According to the structure, five positively charged residues ($a = 5$) of IRF1 DBD, K43, R64, K75, K78, and R99, form salt bridges with four DNA phosphates ($b = 4$). Residues R99 and K43 contact the same phosphate. It appears from the crystal structure that only residue K75 is capable of

forming a salt bridge ($c = 1$) with a negatively charged residue, D73, in the free protein.

Due to the high energy of dehydration, fluoride ions do not interact strongly with protein (*33*). To simplify our analysis, we assume δ_1 is zero, which has been done previously (*20–22, 34*). Thus subtracting eq 10 from eq 11 gives a δ_2 of 3.2, and hence, δ_2 equals 0.64. From the standard errors of the slopes, the error of δ_2 is 0.16. Similar analysis of eqs 10 and 12 gives a δ_3 of 1.02 ± 0.14. When these values are substituted into eq 4, ψ is calculated to be 0.65 with an error of 0.05. Equation 5 then gives a φ of 1.16 ± 0.64.

The number of cations released from the DNA, q, is approximately 2.6. The number of cations taken up by IRF1 DBD, m, is approximately 1.2. The number of anions released from the protein, n, is approximately 3.2 for chloride. The number of bromides released from IRF1 DBD is approximately 5.1.

We solved for δ_2 and δ_3 using the equations describing the salt concentration dependence of binding of the mutant D73A, eqs 10−12. The equations describing the salt concentration dependence of binding of the mutant K75A, eqs 13−15, or those of the mutant D73A/K75A, eqs 16−18, could have been used instead. Using eqs 13−15, δ_2 is 0.65 ± 0.075 and δ_3 is 0.9 ± 0.1. Using eqs 16−18, δ_2 is 0.78 ± 0.075 and δ_3 is 0.98 ± 0.1. Therefore, the values of the unknowns δ_2 and δ_3 do not depend strongly on the equations used to solve for them. This is also true for the determination of ψ and φ. When these estimated values for the unknowns are substituted into eqs 4−18, close agreement of the left-hand terms of the equations with the experimentally determined values on the right-hand side lends reliability to the solutions obtained.

DISCUSSION

In this work, we describe the binding of IRF1 DBD to its DNA target sequence over a range of salt concentrations for six salts, NaF, KF, NaCl, KCl, NaBr, and KBr. We mutated charged residues, which form salt bridges with DNA phosphates according to the crystal structure of the IRF1 DBD−DNA complex. The mutations alter the measured stoichiometry of ion release, providing evidence of ion condensation on IRF1 DBD and its DNA binding site. The salt concentration and type dependencies of the K_d values of the mutants relative to that of WT IRF1 DBD also indicate how certain charged residues are neutralized in the free protein, demonstrating whether it is by solution ions or by a salt bridge with a neighboring residue. Using this information, we determined the number, type, and degree of condensation of ions around DNA phosphates and charged protein residues.

Sodium and Potassium Affect IRF1 DBD−DNA Equilibrium. The salt concentration dependencies of IRF1 DBD binding to DNA are similar in the presence of sodium and potassium salts with the same anion (Figure 2 and Table 1). According to eq 2, this result means that similar net numbers of sodium and potassium ions are released during complex formation. Others have also reported that similar numbers of sodium and potassium ions are released upon DNA binding for Ku (*21*), single-stranded binding protein (*34*), and engrailed (*20*).

While cation type does not significantly affect the salt concentration dependence of the K_d of IRF1 DBD for DNA, cation type does affect the magnitude of the K_d. Binding is tighter in potassium salts than in sodium salts with the same anion (Figure 2). This difference may be due to uptake of cations by the protein and/or release of cations from the DNA. Because cations can interact with both protein and DNA, we cannot discern the relative contributions of the interactions responsible for the cation type effect on the K_d. Experiments using X-ray crystallography (*37*), molecular dynamics simulations (*38*), and NMR (*39*) have demonstrated differences in the location but not the number of Na^+ and K^+ ions around DNA, which are consistent with our data that indicate that cation type affects the magnitude of the K_d but not the salt concentration dependence of the K_d. Differences in the location and affinity of cations for protein surfaces have also been observed using molecular dynamics (*40, 41*) and X-ray crystallography (*42*).

Cation Localization around DNA. To determine the degree of condensation or effective concentration of cations around DNA phosphates, Record and colleagues (*10*) measured the salt concentration dependence of binding of polylysine peptides of varying lengths to DNA. Assuming there was no release of anions from the polylysine peptide upon its binding DNA, Record and colleagues determined the localization of cations around the phosphates of native DNA to be 0.88 (*10*). Since then many salt concentration dependence studies (*11, 13–18, 21, 24, 25, 34*) have used this value for ψ. More recently, grand canonical Monte Carlo simulations were used to predict the local ion effects on binding of an oligocation with eight positive charges to DNA oligomers of varying lengths (*43*). The degree of localization or condensation that is measured depends strongly on the DNA length.

The work reported here appears to be the first measurement of the localization of cations around DNA phosphates from the local ion effects on binding of a globular protein without assuming that anions are not released from the protein. We calculate ψ to be 0.65 ± 0.05.

Instead of local ion effects from the neutralization of phosphates, global ion redistribution has been proposed to give rise to the salt concentration dependence of protein−DNA interactions (*26*). That is, in a protein−DNA complex, the large negative electrostatic potential near the DNA excludes anions from the protein's surface, and the protein's presence excludes cations near the DNA (*26*). In support of this proposal, Misra and colleagues compared the reported salt concentration dependence of λcI repressor binding and *Eco*RI binding with their nonlinear Poisson–Boltzmann calculations of ion concentrations around the two proteins.

Misra et al. (*26*) suggest residue E34K in λcI repressor does not make direct contacts with DNA, and hence, the difference between the salt concentration dependence of binding of this mutant λcI repressor and wild-type repressor cannot be explained by local ion effects. They also propose that the difference between the salt concentration dependence of binding of a deletion mutant of the first 12 residues of the N-terminal region of *Eco*RI endonuclease relative to that of wild type cannot be explained by local ion effects. The papers cited by Misra et al. do not, however, support their proposals. On the basis of structural evidence, Nelson and Sauer (*44*) reported that residue E34K in λcI repressor forms

a salt bridge with DNA, and this is consistent with an increased salt concentration dependence of the mutant relative to that of the wild-type λcI repressor. Similarly, Jen-Jacobson et al. (*45*) reported that there is unpublished structural evidence that Arg9 in the N-terminal region of *Eco*RI endonuclease makes a direct contact with the DNA, which is consistent with a decreased salt concentration dependence of a deletion mutant of the N-terminal region relative to that of the wild-type protein.

Our data appear to be well described in terms of local ion effects. For example, the D73A mutant takes up one fewer sodium ion and releases one more chloride ion relative to WT IRF1 DBD, which explains why the salt concentration dependence of binding of the D73A mutant protein is approximately 2 units greater than that of WT IRF1 DBD. Local ion effects can also simply explain why the salt concentration dependencies for WT IRF1 DBD and the mutant proteins K75A and D73A/K75A in buffer containing NaCl are approximately equal. That is, the contribution to the salt concentration dependence from the cation displaced from the phosphate that interacts with K75 in WT IRF1 DBD is offset by the cation taken up by D73, and interaction of an ion with residues 73 and 75 does not contribute to the salt concentration dependence in mutant proteins D73A and D73A/K75A.

Anions Interact Specifically with IRF1 DBD. The salt concentration dependencies of binding differ significantly when the anion is changed (Figure 2 and Table 1). This indicates that when the anion identity is changed, different numbers of solution anions are released from WT IRF1 DBD upon its binding to DNA. This effect must arise from the interaction of anions with the protein because anions do not interact with DNA. Evidence for the release of anions from other DNA binding proteins has been observed previously; specifically, the equilibrium binding constants of single-strand binding protein (*34*), Ku (*21*), engrailed (*20*), and T4 gene 32 protein (*22*) are sensitive to anion identity.

In addition to affecting the salt concentration dependence of the K_d of IRF1 DBD for DNA, anion type also affects the magnitude of the K_d. DNA binding is tightest in fluoride salts, weaker in chloride salts, and weakest in bromide salts for WT IRF1 DBD (Figure 2). This order of anions (F^-, Cl^-, Br^-) follows the Hofmeister series and probably reflects the relative strengths of interactions of the ions with IRF1 DBD. Small ions, like fluoride, have organized, tightly held water shells, whereas larger ions, like bromide, hold water less tightly (*46*). As a result, fluoride interacts weakly with protein, and thus, its release from protein does not contribute to the salt concentration dependence of binding (*27*). Tight binding of IRF1 DBD to DNA in buffer containing fluoride results from a weak interaction between fluoride and the protein. It requires less energy to dehydrate a larger ion, like bromide; therefore, it can interact more strongly with protein, and its release from the protein contributes to the salt concentration dependence of binding. That is, the weak binding of IRF1 DBD to DNA in buffer containing bromide results from a strong interaction between bromide and the protein. Our calculated degrees of condensation of chloride and bromide ions per basic residue of IRF1 DBD that forms a salt bridge with a DNA phosphate (0.64 ± 0.16 and 1.02 ± 0.14, respectively) are consistent with the anion order of the Hofmeister series. The fact that these numbers are less than and close to 1 also lends credibility to their values.

IRF1 DBD Also Takes Up Cations upon Binding DNA. The salt concentration dependence of the K_d of the D73A mutant for DNA relative to that of WT IRF1 DBD is consistent with the uptake of a cation by the protein. We predict and provide evidence that D73 and K75 form a salt bridge in IRF1 DBD in buffer containing chloride or bromide salt. The D73A mutant has a greater salt concentration dependence for binding relative to WT IRF1 DBD in buffer containing chloride or bromide salt (Table 1). This indicates that the mutant protein takes up fewer ions than WT IRF1 DBD. An increase in the slope by approximately 2 in buffer containing NaCl of the D73A mutant relative to WT indicates that one less sodium ion is taken up and one more chloride ion is released from the mutant protein. Hence, we can conclude that in WT IRF1 DBD residue D73 takes up a sodium ion in buffer containing chloride or bromide salt. Data consistent with uptake of cation by other DNA binding proteins, nonlinearity over large ranges of salt concentration of log–log plots of the binding constant versus salt concentration, have been published for CAP (*24*), single-strand binding protein (*34*), papillomavirus E2 proteins BPV-E2/D and HPV16-E2/D (*25*), AraC (*23*), and T4 gene 32 protein (*22*).

Through the use of mutant proteins and fluoride-containing buffers, we were able to calculate the effective concentration of cations around negatively charged residues of IRF1 DBD, φ, to be 1.16 ± 0.64. The fact that the most probable value of φ is greater than one likely results from the relatively high value of its error.

Aggregation at Low Ionic Strengths. In preliminary experiments, we observed that the apparent strength of binding of IRF1 DBD to its DNA binding site increased as the salt concentration increased from 25 to 200 mM (data not shown). This anomalous behavior, unusually high anisotropy values, and ultracentrifugation data (data not shown) are all consistent with aggregation of IRF1 DBD at protein concentrations above 1 μM and salt concentrations below 200 mM. The binding strength decreased as the salt concentration increased above 200 mM (Table 1), as predicted by the polyelectrolyte effect. Therefore, in this paper, we present data collected at salt concentrations only at and above 200 mM. Nonlinearity in the salt concentration dependence of binding over large ranges of salt concentration has been observed previously and ascribed to association of ion with the protein (*23, 24*). Protein aggregation at low salt concentrations is a potential alternative explanation of nonlinearity in the salt concentration dependence of binding in such experiments, and data suggest that this is the case in this study.

Role of Salt Bridges in IRF1 DBD. Residues D73 and K75 form a salt bridge in buffer containing chloride or bromide salt but not in buffer containing fluoride salt. It is unclear why. The fact that fluoride does not interact strongly with proteins cannot explain this difference because our experiments are capable of detecting the difference in the salt concentration dependencies of binding for the salt bridge case and the counterion case in buffer containing NaF. A possible explanation for the different forms of neutralization in NaF and NaCl is that it is energetically more costly to condense two ions, Na^+ and Cl^-, on residues D73 and K75 in the free

protein than it is to form a salt bridge between the two residues. Alternatively, perhaps in buffer containing NaCl or NaBr, K75 is in a conformation that positions it close to D73 and allows the formation of a salt bridge; however, in buffer containing NaF, K75 and D73 are not situated to form a salt bridge, so instead, these residues are neutralized by counterions. In principle, this hypothesis could be tested by a denaturation experiment in fluoride-containing buffer. A different conformation of residues D73 and K75 in different salts supports a global ion redistribution model, but our data are more easily explained by local ion effects.

Our salt concentration dependencies of binding, which are measured between 200 and 650 mM, indicate that D73 and K75 form a salt bridge in free IRF1 DBD in buffer containing chloride or bromide salt (Table 1). This finding opposes the fact that some salt bridges in proteins persist at low salt concentrations (<0.1 M) and tend to be disrupted at high salt concentrations (*47*).

According to structural information (*35, 48*), several other DNA binding proteins such as integration host factor, single-strand binding protein, lac repressor, DNA gyrase, the nucleosomal proteins, RNA polymerase core protein, c-Myb, and HU contain cationic residues that participate in salt bridges in the free protein and interact with DNA phosphates in the complex. If two residues have formed a salt bridge, an anion would not be displaced from the positively charged residue upon DNA binding. Also, the negatively charged residue would take up a cation when the positively charged residue interacts with the DNA. Releasing fewer ions and taking up more ions reduce the protein's salt concentration dependence of binding. Mechanisms that reduce the salt concentration dependence of binding are important for a cell to avoid large fluctuations in gene regulation in response to changes in cellular conditions (*23, 49, 50*).

In conclusion, the studies presented in this work demonstrate ions condense on free IRF1 DBD in addition to DNA. We measured the number, type, and degree of condensation of ions around this protein and DNA. Upon formation of the IRF1 DBD−DNA complex, between two and three cations are released from the DNA binding site, one cation is taken up by IRF1 DBD, and three chloride or five bromide ions are released from IRF1 DBD. Specifically, residue D73 takes up a cation, and residues R64 and K75 release anions when IRF1 DBD binds to DNA in buffer containing fluoride salt. The salt concentration dependencies of four mutant proteins relative to that of the WT protein indicate that in free IRF1 DBD, residue R64 is neutralized by a solution ion and residues D73 and K75 form a salt bridge in buffer containing chloride or bromide salt. We calculated the average degree of condensation of cations per DNA phosphate is 0.65 ± 0.05; the average degree of condensation of cations per acidic residue is 1.16 ± 0.64, and the average degrees of condensation of chloride and bromide ions per basic residue are 0.64 ± 0.16 and 1.02 ± 0.14, respectively. We conjecture that, like IRF1 DBD, other DNA binding proteins have a mixture of basic residues that form salt bridges and basic residues that are neutralized by solution ions. We also predict that the degree of condensation of ions around acidic and basic residues in other DNA binding proteins and DNA phosphates will be similar to those we measured for IRF1 DBD and its DNA binding site.

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