Preferential Interactions of the *Escherichia coli* LexA Repressor with Anions and Protons Are Coupled to Binding the *RecA* Operator†

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ABSTRACT: The binding of *Escherichia coli* LexA repressor to the *recA* operator was examined as a function of the concentration of NaCl, KCl, NaF, and $MgCl_2$ at pH 7.5, 21 °C. The effects of pH at 100 mM NaCl were also examined. Changes both in the qualitative appearance of the binding isotherms and in the magnitude of the apparent binding affinity with changes in solution conditions suggest that binding of anions and protons by LexA repressor is linked to oligomerization and/or operator binding. Binding of LexA repressor to the *recA* operator in the presence of NaCl ranging from 25 to 400 mM at picomolar DNA concentration showed a broad, apparently noncooperative, binding isotherm. Binding of LexA repressor in NaF at the same [DNA] yielded binding isotherms with a narrow transition, reflecting an apparently cooperative binding process. Also, the apparent binding affinity was weaker in NaF than in NaCl. Furthermore, the binding affinity and also the apparent binding mode, cooperative vs noncooperative, were pH dependent. The binding affinity of LexA repressor for operator was greatest near neutral pH. The apparent binding mode was noncooperative at pH 7−9 but was cooperative at pH 6 or 9.3. These observations suggest that the specific cation and anion composition and concentrations must be considered in understanding the details of regulation of the SOS system.

The *Escherichia coli* LexA repressor coordinately controls about 20 unlinked genes comprising the bacterial SOS response, an inducible DNA repair system. These genes are necessary for functions as diverse as mutagenesis, DNA repair, recombination, cell division, and prophage induction (Little & Mount, 1982; Walker, 1985). LexA repressor is a 202 amino acid monomer with a 2-domain structure (Little & Mount, 1982; Schnarr et al., 1991; Little, 1993). The N-terminal domain (amino acids 1−84) is the DNA-binding domain (Little & Hill, 1985; Brent & Ptashne, 1985; Hurstel et al., 1986), while the C-terminal domain is called the oligomerization domain (Schnarr et al., 1988). The SOS response is activated upon autoproteolysis of LexA repressor into its two separate domains (Little, 1984). Induction apparently results from the reduction in the specific affinity of LexA repressor for its operator sites after proteolytic cleavage. The isolated N-terminal DNA-binding domain binds to operator DNA fragments 10−1000-fold more weakly than does the intact protein (Bertrand-Burggraf et al., 1987; Kim & Little, 1992).

The operator sites of LexA repressor have the consensus sequence 5′CTGTATATATATACAG3′. In this sequence, the exterior 4 base pairs (CTGT) were shown to be most critical for recognition and binding by LexA repressor (Wertman & Mount, 1985). Most genes within the SOS regulon possess single operator sites in their regulatory control regions; however, several have two operator sites with variable spacing between their dyadic centers, and the *recN* gene has been shown to have three sites (Schnarr et al., 1991; Rostas et al., 1987). The position of the operators relative to the promoters is highly variable among the SOS operons (Schnarr et al., 1991). These facts suggest a highly intricate regulatory scheme optimizes the cellular response to DNA damage by adjusting the balance of the SOS proteins induced.

The operator-bound form of LexA repressor is thought to be the dimer. *In vivo* experiments demonstrated that at least one LexA monomer interacted with each operator half-site (Thliveris et al., 1991). However, LexA repressor has been reported to dimerize only weakly in the absence of DNA, with a dissociation constant of 15−50 μM (Schnarr et al., 1985; Kim & Little, 1992). Cellular concentrations of LexA repressor monomer have been estimated to be 1−2 μM (Moreau, 1987; Sassanfar & Roberts, 1990; Dri & Moreau, 1994) with only 20% free in solution. Therefore, most of the unbound cellular LexA repressor would be monomeric. Operator binding experiments performed *in vitro* typically use protein concentrations in the picomolar to nanomolar range, where most of the LexA repressor would also be predicted to be monomeric in the absence of DNA. Monomer binding to the operator followed by dimer assembly on the DNA was inferred from experiments *in vitro* in which operator half-sites were bound by LexA repressor with lower affinity than full sites (Kim & Little, 1992).

Binding by LexA repressor to a number of operators has been studied using several techniques, but usually at only one solution condition (Brent & Ptashne, 1981; Little et al., 1981; Ebina et al., 1983; Kitagawa et al., 1985; Schnarr et al., 1985; Granger-Schnarr et al., 1986; Lloubes et al., 1986; Bertrand-Burggraf et al., 1987; Rostas et al., 1987; Lloubes et al., 1991; Kim & Little, 1992; Oertel-Buchheit et al., 1992; Oertel-Buchheit et al., 1993). However, the stability and specificity of protein−nucleic acid complexes generally vary with changes in environmental conditions (Record et al., 1978; Record & Spolar, 1990; Lohman & Mascotti, 1992). We are therefore studying the thermodynamics of DNA

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binding by LexA repressor at a variety of solution conditions in order to understand how changes in environmental conditions will affect cellular regulation of the *E. coli* SOS response.

In this work, we examined binding of LexA repressor to the *recA* operator using the gel mobility shift assay and determined the stoichiometry of binding by fluorescence quenching. We observed that anion type and pH have profound effects on both the strength of binding and the apparent cooperativity of binding of LexA repressor to the *recA* operator. Our results suggest that binding of anions and protons by LexA repressor is linked to oligomerization and/or operator binding by the protein. *In vivo* effects of extracellular pH on SOS induction have been observed and shown to arise due to transient loss of intracellular pH homeostasis (Schuldiner et al., 1986; Dri & Moreau, 1994). We suggest that the derepression of the SOS response observed at acidic pH might arise, at least in part, due to loss of selectivity of DNA binding by LexA repressor.

EXPERIMENTAL PROCEDURES

Buffers. The standard buffer for the binding reactions was buffer T (pH 7.5 at 21 °C) containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] 1 mM EDTA, 0.04 mg/mL BSA, 5% glycerol, and different concentrations of NaCl, NaF, KCl, or $MgCl_2$ as indicated in the text. The pH of buffer T did not vary by more than ±0.1 over the range of salt concentrations examined. In some experiments, buffer T containing 100 mM NaCl was titrated to other pH values as indicated. Buffer P (pH 6.0, 6.9, 7.4, or 7.9) was 10 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, 0.04 mg/mL BSA, and 7% glycerol. Buffer C (pH 2.9, 3.9, 4.9, or 5.9) was 10 mM sodium citrate, 100 mM NaCl, 1 mM EDTA, 0.04 mg/mL BSA, and 7% glycerol. Buffer M (pH 5.1, 6.0, or 7.0) was 10 mM MES, 100 mM NaCl, 1 mM EDTA, 0.04 mg/mL BSA, and 7% glycerol. Buffer L was 10 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA, 0.04 mg/mL BSA, 5% glycerol, and 50 or 100 mM KCl as indicated.

LexA Protein. LexA repressor was overexpressed using *E. coli* strain JL652 (from John Little, University of Arizona). Growth of the bacteria and purification of the protein followed previously published protocols (Little, 1984; Slilaty et al., 1986). The purified protein was stored in 10 mM Pipes (pH 7.0), 200 mM NaCl, 0.1 mM EDTA, and 50% glycerol at −20 °C. Protein concentrations were determined spectrophotometrically using an extinction coefficient at 280 nm of 7300 M^{-1} cm^{-1}. The protein was assumed to be 100% active in operator binding in these studies.

Nucleic Acids. The 176 bp *Eco*RI−*Hin*dIII *recA* operator-containing DNA fragment in pJWL5 (gift of John Little, University of Arizona) was isolated and cloned into pUC119 using conventional methods (Sambrook et al., 1989). Plasmid was grown in DH5α cells in LB medium and purified using Qiagen plasmid tips (Qiagen Inc., CA). The 176 bp operator-containing fragment was obtained by digestion with *Eco*RI and *Hin*dIII, purified by electrophoresis on a nondenaturing 5% polyacrylamide gel, and radioactively labeled with ^{32}P at the 5′ ends using T4 polynucleotide kinase (Sambrook et al., 1989). The radiolabeled 176 bp fragment was further purified by electrophoresis on a nondenaturing 5−15% polyacrylamide gradient gel and then stored in 10 mM Tris (pH 8.0 at 21 °C), 0.1 mM EDTA at 4 °C. Concentrations of the DNA fragment were determined spectrophotometrically using an extinction coefficient at 260 nm of 2.34×10^6 M^{-1} (molecule) cm^{-1}.

The 35 bp *recA* operator-containing oligonucleotide used in the fluorescence titrations had the sequence: $^{5'}$CCCT-TGATACTGTATGAGCATACAGTATAATTCCC$^{3'}$ (upper strand). The upper strand was labeled at the 5′ end with fluorescein and was a gift, synthesized in the lab of Christine Chow (Wayne State University). It was purified by 15% denaturing polyacrylamide gel electrophoresis. The lower strand was purchased from Ransom Hill Bioscience. Duplex DNA was formed by annealing equimolar quantities of both strands in 100 mM NaCl, 10 mM Tris (pH 8.0), heating to 70 °C, and cooling. The duplex DNA was purified from excess single-stranded oligonucleotide by electrophoresis through a 15% native polyacrylamide gel and subsequently stored in 10 mM Tris (pH 7.5), 200 mM NaCl at −20 °C. Concentrations were determined spectrophotometrically using an extinction coefficient at 260 nm of 692 500 M^{-1} (molecule) cm^{-1}.

Fluorescence Titrations. Fluorescence measurements were made using a Spex Fluoromax. Stoichiometric titrations were performed at room temperature (21 ± 1 °C) in buffer T including 100 mM NaCl, 100 mM NaF, or 5 mM $MgCl_2$, but lacking BSA. The 35 bp fluorescein end-labeled oligonucleotide was used at an initial concentration of 0.1 μM. The excitation wavelength was 492 nm with a band-pass of 1.7 nm (0.4 mm slit width), while emission was monitored at 520 nm with a band-pass of 3.2 nm (0.8 mm slit width). A 500 nm cuton filter (Spex KV500) was inserted into the emission path. Titration of the oligonucleotide was performed by the sequential addition of small volumes of 2.0 μM LexA equilibrated to room temperature in the appropriate buffer. Mixing was performed manually with a polystyrene stirrer after each addition of protein. Equilibration of the samples required at least 2 min. Emission spectra, taken any time after equilibration was complete, were identical. An analogous titration of protein into buffer without oligonucleotide was used to determine background fluorescence for each titration point. Fluorescence intensities were also corrected for volume changes using the following equation: $F_{i,\mathrm{corr}} = (F_{i,\mathrm{obs}} - F_{i,\mathrm{bl}})V_i/V_o$, where $F_{i,\mathrm{corr}}$ is the corrected fluorescence intensity for point i of the titration, $F_{i,\mathrm{obs}}$ is the observed intensity for point i, $F_{i,\mathrm{bl}}$ is the observed fluorescence intensity of the blank, V_i is the volume after the ith addition, and V_o is the initial volume of the titration. Two to four titrations were performed at each condition.

Gel Mobility Shift Assay (Garner & Revzin, 1981; Fried & Crothers, 1981; Fried, 1989; Carey, 1991). Most binding titrations were performed in buffer T at room temperature (21 ± 2 °C) with added salt as indicated. The 25 μL reaction mixtures contained 31 pM *recA* operator DNA. LexA repressor was serially diluted immediately before use into reaction buffer at room temperature to span the necessary concentration range (0−55 nM) for the titration reactions. Mixing was performed by repeated pipetting. The reaction was equilibrated for 20 min. Experiments at 100 mM NaCl

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; bp, base pair; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

indicated that the binding equilibrium was achieved in 30 s and prolonged reaction time (over 20 min) did not have any effect on the equilibrium position. The bound and free DNA fragments were resolved by native gel electrophoresis in 8% polyacrylamide using 10 mM Tris-HCl, pH 7.5, 1 mM EDTA as the buffer for gel casting and running. A Hoeffer SE500 apparatus was used for electrophoresis; usually two gels were run simultaneously on each unit. Gels were prerun at 150 V for 30 min; then samples were loaded while the gel was running. Each gel was run for 3−4 h at 150 V at room temperature with constant recirculation of the buffer. Typical currents were about 25−27 mA per 1.5 mm thick gel (14 × 16 cm). Wet gels were exposed to a storage phosphor screen and analyzed for bound and free DNA using a Molecular Dynamics Phosphorimager. At each experimental condition, 2−8 titrations were performed.

For titrations under varying H^+ concentrations, we employed four overlapping buffer systems covering the pH range 2.9−9.3: buffer C (pH 2.9−5.9), buffer M (pH 5.1−7.0), buffer P (pH 6.0−7.9), and buffer T (pH 7.1−9.3). Each buffer contained 100 mM NaCl. Other reaction protocols were identical to those mentioned above.

Data Analysis. The available literature on the assembly of LexA repressor indicates that in the absence of DNA the protein is monomeric at the concentrations we have used (Schnarr et al., 1985; Kim & Little, 1992). There is apparently little, if any, change in the oligomerization constants with changes in salt or pH (Schnarr et al., 1985; Hurstel et al., 1990). Formulation of a detailed binding mechanism is precluded by the lack of data about oligomerization at the specific solution conditions we have examined. We have therefore used the apparent binding affinity (K_{app}), defined as the inverse of the concentration of LexA repressor at 50% saturation of the operator, to compare relative binding affinities as a function of solution conditions. Rather than estimating K_{app} from a visual inspection of the data, K_{app} was determined by fitting the binding isotherms either to the Langmuir isotherm for independent site binding or to an equation for a model in which dimerization of monomers is coupled to operator binding. Data were analyzed using PSIplot (Poly Software International, Salt Lake City, UT). The Langmuir isotherm has the form $\theta = K_{app}P_T/(1 + K_{app}P_T)$, where θ is the fraction of DNA bound and P_T is the total LexA concentration in the reaction. The equation used to fit data to the model with coupled dimerization required for binding was $\theta = K_{app}^2P_T^2/(1 + K_{app}^2P_T^2)$. The assumption that total protein concentration equals free protein concentration is valid for most of the titrations performed. However, for a few titrations performed at conditions with the tightest affinity (e.g., 5 mM $MgCl_2$), this assumption breaks down at the lower protein concentrations used and K_{app} represents only a lower limit on the apparent affinity.

Theory of Salt Effects. The equilibrium interactions of proteins with nucleic acids are often highly sensitive to changes in the salt concentration (Record et al., 1978; Record & Spolar, 1990; Lohman & Mascotti, 1992). The molecular origin of this sensitivity can arise from a number of sources. The polyelectrolyte effect, arising because of the linear polyanionic nature of DNA, is often one major cause. The high linear negative charge density results in accumulation of counterions in the vicinity of the helix. Binding of simple oligocations (that do not themselves have any specific interactions with other small ions in the solution) will result in release of a characteristic number of counterions from the DNA related to the valence of the oligocation (z) and the fractional extent of charge neutralization due to counterion accumulation on the DNA (ψ) (deHaseth et al., 1977; Record et al., 1978; Record & Spolar, 1990). This results in a signature dependence of the equilibrium constant (K) of the binding reaction upon the salt concentration; log K should decrease linearly with increasing log [salt]. The slope of this line, s(K), will have the form:

$$s(K) = \delta \log K/\delta \log [\text{salt}] = -z\psi$$

and represent the net number of cations released upon complex formation. If the ligand binding to the DNA is a protein, the salt dependence of the equilibrium constant for complex formation will have additional contributions if preferential interactions of anions, cations, or water with the protein are affected by the interaction with the DNA**.** The dependence of log K on the log of the monovalent salt concentration need not be linear in this case, but the instantaneous slope at any given concentration of salt will represent the net amount of ion release accompanying the reaction and can be written as

$$s(K) = -z\psi - \Delta C - \Delta A - \Delta W$$

where ΔC represents the number of cations released from the protein on binding to the DNA, ΔA represents the number of anions released from the protein on binding to the DNA, and ΔW represents released waters of hydration.

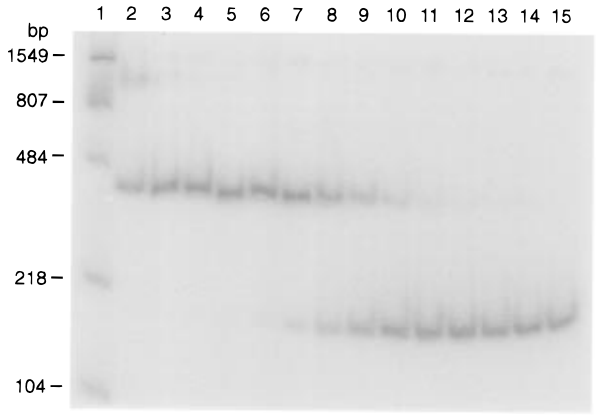

FIGURE 1: Free and bound operator-containing DNA fragments were separated using the gel mobility shift assay as described under Experimental Procedures. This experiment was performed at 21 °C in buffer T with 150 mM NaCl using 31 pM DNA. Lane 1 contains DNA size markers. Reactions in lanes 2−14 contained LexA repressor as follows: 55 nM, 25 nM, 11 nM, 5.0 nM, 2.3 nM, 1.0 nM, 460 pM, 210 pM, 93 pM, 42 pM, 19 pM, 8.5 pM, and 3.8 pM. The reaction in lane 15 contained only DNA.

RESULTS

Typically, the binding of proteins to nucleic acids is sensitive to the ionic environment of the solution. To monitor equilibrium binding as a function of salt at pH 7.5, titrations of 31 pM *recA* operator with LexA repressor were performed at room temperature (21 °C) in buffer T and analyzed by the gel mobility shift assay. Figure 1 shows a gel of a titration at 150 mM NaCl. The primary complex made between LexA repressor and the 176 bp *recA* operator fragment migrated with the mobility of a 340 bp fragment of DNA. At the highest protein concentration used in this

titration (55 nM), a second complex of very low mobility was observed. This product is probably the complex formed when LexA repressor fills all available nonspecific binding sites on the fragment.

Measurement of equilibrium constants using the gel mobility shift assay requires that there be no perturbation of the equilibrium by the electrophoresis process. This requirement appears to be valid for the experiments presented here based on three different lines of evidence. First, the data in NaCl at pH 7.5 were collected using two different electrophoresis conditions. In addition to our standard electrophoresis protocol (see Experimental Procedures), some experiments were analyzed by electrophoresis on 0.75 mm gels of 5% polyacrylamide run at 4 °C for 2 h at 150 V with buffer recirculation. Results obtained in both gel systems were identical within the error of the method over the entire range of NaCl concentrations examined. Second, the data for a number of experiments at 100, 200, and 300 mM NaCl were analyzed in two ways to assess whether significant complex dissociation occurred during electrophoresis. Binding isotherms were determined separately using either the complexed DNA or the free DNA bands and then compared (Senear & Brenowitz, 1991). The values of K_{app} calculated from the two sets of isotherms (data not shown) were indistinguishable within the precision of the data at each of the three salt concentrations. Apparent binding affinities were not systematically larger when determined from the free DNA bands, suggesting that any dissociation during electrophoresis was not detectable. Finally, to compare our measurements of K_{app} directly with previously published values, we performed several experiments in buffer L, analogous to the buffer used by Kim and Little (1992) in DNase I footprinting experiments with the *recA* operator. At 50 mM KCl, $K_{app} = (1.3 \pm 0.6) \times 10^{10}$ M^{-1} and at 100 mM KCl, $K_{app} = (7.2 \pm 0.8) \times 10^{9}$ M^{-1}; the isotherms appeared noncooperative at both conditions. The values of K_{app} we determined were about 6-fold larger than those determined by footprinting, possibly due to the presence of 2.4 μg/mL competitor calf thymus DNA in the footprinting reactions which could lower the apparent affinity measured by that technique. Thus, the equilibrium for complex formation does not appear to be significantly perturbed for this system at the conditions at which we performed the gel mobility shift assay.

Binding Isotherms in NaCl, KCl, and $MgCl_2$ Appear Noncooperative. Operator binding by LexA repressor was examined at NaCl concentrations between 25 and 400 mM. Representative isotherms for 50, 100, 200, and 300 mM NaCl are shown in Figure 2A. At higher salt, the binding of LexA repressor to the operator was weakened as expected. The breadth of the binding transition was roughly 2 decades of protein concentration, suggesting a noncooperative binding process. The data were fit reasonably well by an independent site binding isotherm. The curves superimposed with the data in Figure 2A assume a 1:1 binding stoichiometry. A limited number of experiments were performed in buffer T using 100−300 mM KCl. Within the error of the method, the results were identical to those in NaCl (data not shown).

The dependence of the apparent affinity constant on the concentration of NaCl is shown in Figure 3. Below 75 mM NaCl (log [NaCl] = −1.12), the affinity appeared to be independent of the salt concentration within the precision of our measurements. At concentrations of 75 mM and higher, log K_{app} decreased linearly with log [NaCl]. The slope of the line describing these data was determined by linear least-squares regression to be $s(K) = -(0.9 \pm 0.2)$, where the error represents the standard deviation of the slope.

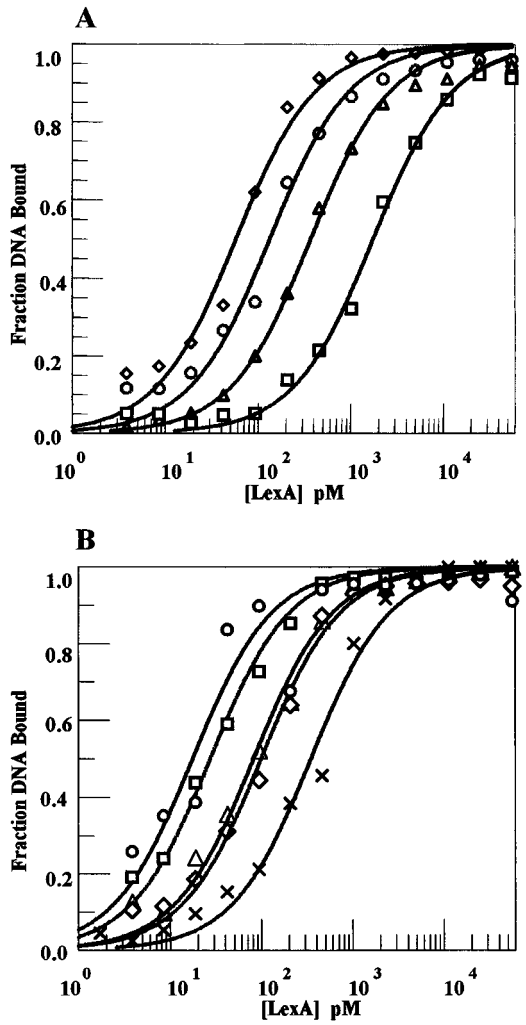

FIGURE 2: Representative binding isotherms determined at 21 °C in salts containing Cl^-. (A) Isotherms determined at 50 (◇), 100 (○), 200 (△), and 300 (□) mM NaCl. (B) Isotherms determined at 5 (○), 10 (□), 20 (△), 50 (◇), and 100 mM (×) $MgCl_2$. The curve through each set of data points represents the best fit to an independent site binding model.

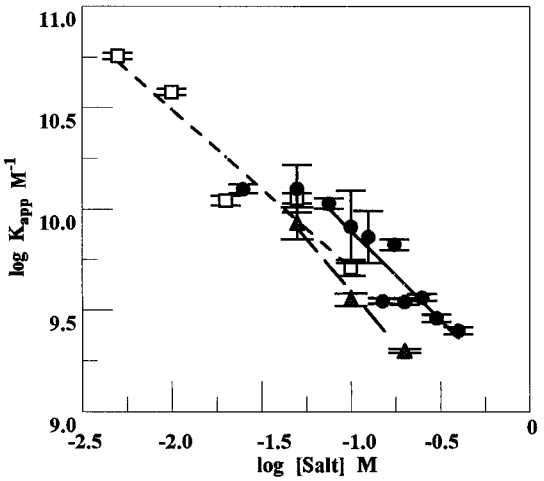

FIGURE 3: Dependence of the apparent binding affinity at 21 °C on the concentration of salt. Data obtained in NaCl (●), $MgCl_2$ (□), and NaF (▲) are shown. Error bars represent the range of values determined from multiple separate isotherms.

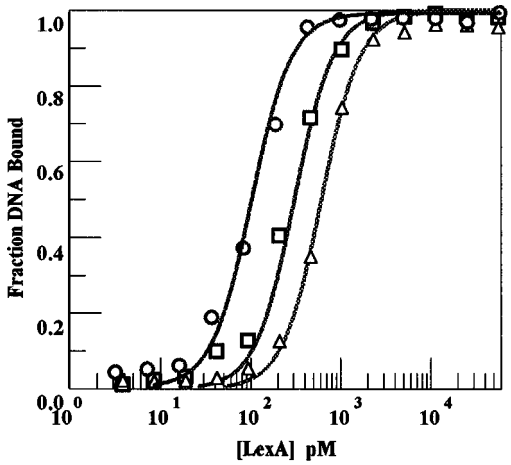

FIGURE 4: Representative binding isotherms determined at 50 (○), 100 (□), and 200 mM (△) NaF at 21 °C. The curves through each set of data represent the best fit to a model with protein dimerization coupled to binding.

One diagnostic test that can help deconvolute the molecular origin of effects of small ions on ligand−nucleic acid interactions is to vary the charge on the cation, while keeping the identity of the anion constant. If the only effect of the cation is due to the polyelectrolyte effect, the slope of a plot of log K_{app} vs log [Mg^{2+}] should be approximately half that seen for the slope of log K_{app} vs log [Na^{+}] (deHaseth et al., 1977). We have performed titrations in 5−100 mM $MgCl_2$. Sample isotherms are shown in Figure 2B. Binding titrations shifted to higher protein concentrations as the $MgCl_2$ concentration increased, and the breadth of the binding transition was again roughly 2 decades of protein concentration. K_{app} for binding to the operator was obtained by fitting to an independent site model. The best fit curves to these data sets are shown in Figure 2B. The dependence of K_{app} on [$MgCl_2$] is shown in Figure 3. The slope of the least-squares line describing these data was determined to be $s(K) = -(0.8 \pm 0.1)$. The dependence of log K_{app} on log [$MgCl_2$] is identical within our experimental error to that found for the dependence of log K_{app} on log [NaCl], suggesting that the polyelectrolyte effect is not the only source of the dependence of K_{app} on salt.

Binding Isotherms Determined in NaF Appear Cooperative. Variation of the anion, while keeping the cation identity constant, is one standard test to determine if the salt dependence of an equilibrium constant has any contribution due to anion binding by the protein. Figure 4 shows binding isotherms obtained in 50, 100, and 200 mM NaF using 31 pM DNA. The shapes of the isotherms were dramatically different from the isotherms in comparable concentrations of NaCl. The transition occurred over a much narrower range of protein concentrations, suggesting that operator binding is cooperative. The data were best fit by a cooperative binding model, such as one in which dimerization of the monomeric LexA repressor is coupled to binding to the operator. The dependence of the apparent binding affinities on NaF concentration is shown in Figure 3. The absolute value of K_{app} was smaller in NaF than in NaCl at any given concentration of salt. The slope for the least-squares line describing these data is $s(K) = -(1.0 \pm 0.1)$. These data, in conjunction with the data in NaCl, suggest that there is a specific effect of anion identity on the binding interaction between LexA repressor and the operator.

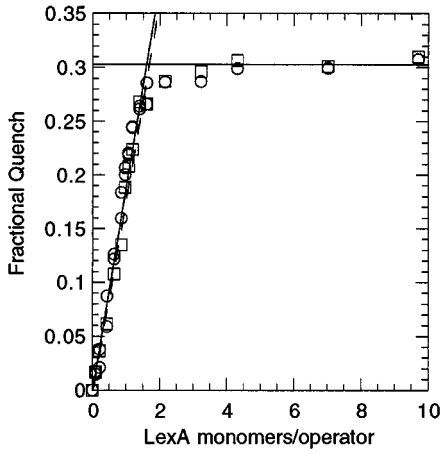

FIGURE 5: Stoichiometric titration of fluorescein-labeled oligonucleotide containing the *recA* operator with LexA repressor at 21 °C in buffer T (minus BSA) with 100 mM NaCl (○) or NaF (□).

Stoichiometry of Binding to the Operator. The stoichiometry of LexA repressor binding to operator was determined at room temperature in buffer T, lacking BSA, at three different salt conditions: 100 mM NaCl, 100 mM NaF, and 5 mM $MgCl_2$. Stoichiometries were determined by titrating a 35 bp fluorescein-labeled oligonucleotide with protein. Upon binding of protein, the maximum fluorescence quenching observed was 30% in all three buffers. Figure 5 shows representative titrations in NaF and in NaCl. Stoichiometries determined in the three salts were identical within the error of the measurements: 2.0 ± 0.3 monomers per operator oligonucleotide in 100 mM NaF, 1.7 ± 0.2 in 5 mM $MgCl_2$, and 1.7 ± 0.2 in 100 mM NaCl. These data show that the binding stoichiometry of the final complex formed does not vary with the identity of the anion.

Strength of Operator Binding and the Apparent Binding Mode Are Dependent on pH. To test whether proton binding affected the interaction of LexA repressor with the *recA* operator, we examined operator binding as a function of pH. Four different buffer systems were used to cover a range of pH from 2.9 to 9.3. Each buffer contained 100 mM NaCl. Since we observed an effect of different anions on the DNA binding of LexA repressor, we tried to control for the presence of the different buffer species by using buffers with some overlap in their buffering regimes. The apparent binding affinity and the apparent cooperativity of binding varied with pH. Representative binding isotherms at room temperature are shown in Figure 6A for pH varying from 4.9 to 9.3 in buffer C (pH 4.9 and 5.9) and buffer T (pH 7−9.3). The isotherms at pH 4.9, 7.6, and 8.9 showed a broad transition, characteristic of noncooperative independent site binding. At pH 5.9 or 9.3, the isotherm transitions were narrow, reflecting a cooperative binding process. The narrow, cooperative transition was seen in all buffer systems near pH 6 (data not shown for buffers P and M). These isotherms were fit by a binding model with coupled protein dimerization. At pH 3.9 (data not shown), the binding transition was very broad (over 3 decades), reflecting apparent negative cooperativity which may be due to protein denaturation since denaturation of the N-terminal domain was reported to begin below pH 5.0 (Lamerichs et al., 1989). At pH 2.9, the DNA had an altered electrophoretic mobility, suggesting that its conformation had changed significantly.

Figure 6B shows the variation in $[LexA]_{50\%}$ as a function of pH for all buffers used. The tightest binding (lowest

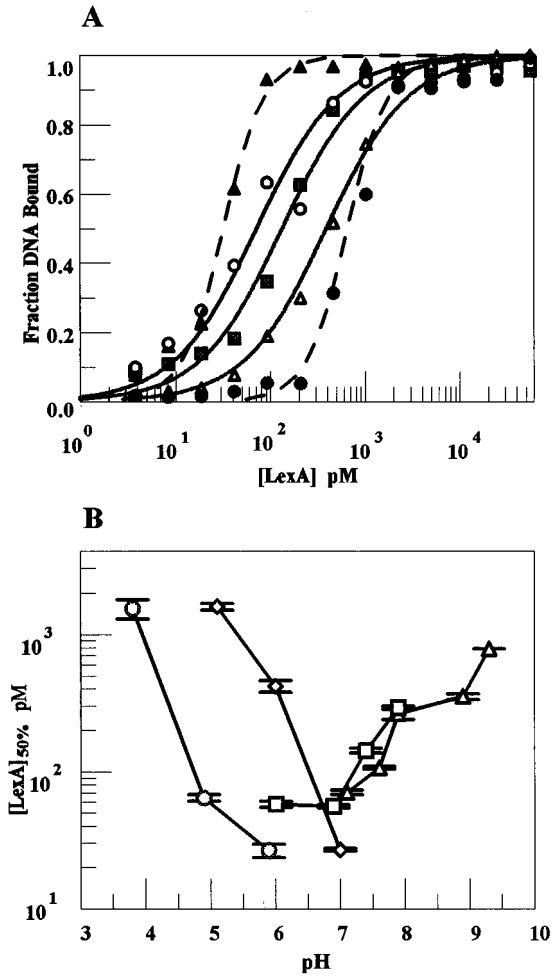

FIGURE 6: Effects of pH on operator binding in 100 mM NaCl at 21 °C. (A) Representative binding isotherms in 10 mM citrate at pH 5.9 (▲) and 4.9 (○) and 10 mM Tris at pH 7.6 (■), 8.9 (△), and 9.3 (●). The curves through the data at pH 5.9 and pH 9.3 are best fits to a coupled dimerization binding model, while those through the other data sets are best fits to an independent site binding model. (B) Dependence of $[LexA]_{50\%}$ for operator binding on pH. Reaction buffers used were buffer C (○) for pH 3.9−5.9, buffer M (◇) for pH 5.1−7.0, buffer P (□) for pH 6.0−7.9, and buffer T (△) for pH 7.1−9.3.

$[LexA]_{50\%}$) was observed in the range of pH 6−7. At lower or higher pH, the affinity weakened (higher $[LexA]_{50\%}$). Although the identity of the buffer affected the magnitude of the $[LexA]_{50\%}$ at a given pH (compare buffers C, P, and M at pH 6 or buffers P, M, and T at pH 7 in Figure 6B), the overall trend that the apparent affinity for the operator decreased at pH values above or below pH 6−7 was seen in each buffer system. Little (1984, 1993) observed that at alkaline pH LexA repressor can undergo autoproteolysis to produce its two separate domains. Autoproteolysis of the protein occurs with a half-life of roughly 2 h at pH 9.5, 20 °C (Slilaty et al., 1986). We have verified by Coomassie Blue stained SDS−PAGE that LexA did not degrade into its two separate domains at any of our buffer conditions during the 20 min reaction time (data not shown).

DISCUSSION

The primary observation of this work is that the monovalent anion identity and protons have a profound effect on the coupling of oligomerization and operator DNA binding by the *E. coli* LexA repressor. This fact is apparent from the changes in the binding isotherms measured in NaCl vs NaF, and as pH is varied from 3.9−9.3. The apparent binding affinity and the breadth of the binding transition as a function of LexA repressor monomer concentration depended on the exact solution conditions.

Binding isotherms obtained in KCl or NaCl appeared little different. This type of observation is consistent with a monovalent cation effect arising solely from the polyelectrolyte effect, with the monovalent cation involved only as a counterion condensed on the DNA. The cations K^+ and Na^+ should be virtually identical in this capacity in their effect on the equilibrium (Record et al., 1978).

The binding isotherms in NaCl and $MgCl_2$ showed broad, apparently noncooperative, binding transitions. Comparison of the salt dependence of the apparent binding affinity in the two salts suggests that there are specific ion effects linked to the overall operator binding reaction. If Na^+ and Mg^{2+} are each functioning only as counterions that partially neutralize the DNA charge, then s(K) in Na^+ should be roughly twice that found in Mg^{2+} (deHaseth et al., 1977). The values of s(K) indicate that a net release of 0.8 ± 0.1 ion accompanies operator complex formation by LexA repressor in $MgCl_2$ while a net release of 0.9 ± 0.2 ion accompanies operator complex formation in NaCl. Since s(K) is indistinguishable in these two salts, processes other than the polyelectrolyte effect must contribute to the salt dependence of the apparent affinity constant. These additional contributions to the observed salt dependence must originate with the protein. Changes in preferential divalent cation binding, preferential anion binding, or hydration might be coupled to oligomerization and/or operator binding by LexA repressor and thereby contribute to the net s(K).

The involvement of linked preferential anion interactions with the protein is indicated by two observations: the change in the apparent cooperativity of operator complex formation and the change in the magnitude of K_{app} when F^- is substituted for Cl^-, with Na^+ as the cation. In a preliminary report of some of our results, we did not observe an effect of F^- vs Cl^- on the binding isotherms at 100 and 250 mM NaX using 3 nM operator fragment and a more limited range of protein concentrations (Shaner & Gaissarian, 1996). At high [DNA], we were unable to detect the significant difference in isotherm shape and apparent affinities between the two monovalent salts that we see at 31 pM operator fragment. The fluoride anion has been found to interact only weakly with proteins (von Hippel & Schleich, 1969). Since F^- binds negligibly to proteins, comparison of s(K) obtained in NaF vs NaCl is often used to dissect the relative contributions of net anion vs cation release to the monovalent salt dependence of the equilibrium constant for binding to DNA (Kowalczykowski et al., 1981; Overman et al., 1988; Lohman & Mascotti, 1992). We saw little difference in s(K) for NaCl vs NaF associated with operator binding on a per monomer basis at our conditions (−0.9 ± 0.2 vs −1.0 ± 0.1), indicating that net ion release is similar for operator complex formation in the two salts even though the magnitude of K_{app} and the apparent cooperativity at low [DNA] vary.

The observation of no apparent cooperativity in the binding isotherms in NaCl, KCl, and $MgCl_2$ might be viewed as somewhat unexpected in light of the measured 2:1 stoichiometry for LexA repressor binding to its operator. At our conditions, the published value for the dimerization dissociation constant, 15−50 μM (Schnarr et al., 1985; Kim &

Little, 1992), predicts that very little dimer should exist in our protein population. The binding isotherms might therefore be expected to show filling of the operator over a much narrower range of protein concentrations if dimers must assemble in solution or on the operator. However, Wong and Lohman (1995) have noted that when protein assembly and DNA binding are linked, the DNA concentration regime used to permit free protein concentration to be approximated by the total protein concentration typically does not produce binding isotherms that readily reveal the coupled assembly of the protein. The change in isotherm shape as the anion is changed from Cl^- to F^- reflects changes in one or more of the equilibrium constants characterizing the linkage of operator binding and oligomerization of LexA repressor. These changes can only result due to linkage of anion binding to oligomerization and/or operator binding. The quantitative analysis of the role of anion binding in the protein oligomerization and DNA-binding equilibria is beyond the scope of this paper and will be addressed elsewhere (Relan and Shaner, in preparation).

Three earlier studies examined the effect of NaCl or KCl on operator binding. Kim and Little (1992) studied binding to the *recA* operator by DNase I footprinting at 22 °C in a Tris buffer (pH 7.4) containing 2.5 mM $MgCl_2$ and 1.5 mM $CaCl_2$. Our results in a similar buffer are in reasonable agreement with their data. Our values of K_{app} are about 6-fold larger in magnitude, and the value of s(K), calculated without correcting for divalent ions (deHaseth et al., 1977), is -1.3 ± 0.2 for Kim and Little (50−200 mM KCl) vs -0.9 ± 0.6 for our data. The other two studies used different operator sequences. The affinity of LexA to the *uvrA* operator was determined by studying the competition between RNA polymerase and LexA repressor with the abortive transcription initiation assay at 37 °C in HEPES (pH 7.9) buffer with 10 mM $MgCl_2$ (Bertrand-Burggraf et al., 1987). Mg^{2+} was treated as a competitor for binding to the DNA to determine that s(K) in NaCl was -4.9 ± 0.9. Our experiments cannot be directly compared to this study since we have observed that temperature affects the binding affinity (Shaner & Gaissarian, 1996; Jenuwine, Relan, and Shaner, unpublished data). The binding of LexA repressor to a 30 bp oligonucleotide containing the consensus operator sequence was studied by nitrocellulose filter binding at 20 °C in 350−650 mM NaCl (Oertel-Buchheit et al., 1992). They obtained a value of s(K) = -5.8 ± 0.4. Our value [s(K) = -0.9 ± 0.2] for the dependence of the apparent binding affinity on [NaCl] differs beyond experimental errors with these latter two determinations. These differences might arise due to the different techniques used, the difference in protein concentrations, the differences in pH and/or [NaCl] range covered, or the difference in operator sequence and fragment size.

Several papers have shown that extracellular pH extremes, both alkaline and acidic, have transient effects on internal cellular pH homeostasis resulting in induction of several LexA repressor-controlled genes (Schuldiner et al., 1986; Dri & Moreau, 1994). Previously, the affinity of nonspecific binding of LexA repressor was seen to vary with pH over the range 5.6−8.0 (Hurstel et al., 1990). We show here that the binding affinity of LexA repressor to the *recA* operator and its apparent binding mode are pH dependent. This is the first *in vitro* demonstration that operator binding by LexA repressor is sensitive to pH.

We observed a bell-shaped curve for the dependence of $[LexA]_{50\%}$ on pH in the range pH 3.9−9.3 (Figure 6B) with maximal affinity at pH 6−7. A similar dependence of binding affinity on pH has been observed for operator binding by *E. coli* Gal repressor (Brenowitz et al., 1990) and target binding by the *D. melanogaster* Ultrabithorax protein (Li et al., 1996). The occurrence of a pH optimum suggests that a minimum of two different titratable functional groups must be involved in operator complex formation. The slopes of the curves above neutrality imply that at 100 mM NaCl *net adsorption* of protons accompanies complex formation with operator in phosphate and Tris. Below pH 7, *net release* of protons accompanies operator complex formation in citrate, phosphate, and MES. Changes in proton binding that result in the observed pH dependence of operator complex formation could be linked to any of the multiple equilibria involved in the observed overall operator-binding reaction. In contrast to our observations on operator binding, the cooperative nonspecific binding of LexA repressor to poly[d(AT)] showed decreased binding affinity with increasing pH at 600 mM NaCl in phosphate for pH 5.6−8.0, indicating net adsorption of protons occurs during formation of nonspecific complexes (Hurstel et al., 1990). We have seen a similar dependence for LexA binding to random sequence DNA (Jenuwine and Shaner, unpublished results). This difference suggests that different functional groups are responsible for the pH effects coupled to nonspecific DNA binding and operator binding by LexA repressor.

The mechanisms by which bacterial gene expression is regulated by internal and external pH are not understood for most genes affected by pH (Olsen, 1993). Comparison of our results with those on nonspecific binding to poly[d(AT)] (Hurstel et al., 1990) or to random-sequence DNA (Jenuwine and Shaner, unpublished results) suggests that one effect of acid pH is to change the selectivity of DNA binding by LexA repressor. Operator binding is tightest around neutral pH and is weakened as the pH is lowered; however, nonspecific binding increases its affinity with decreasing pH. Thus, as the pH is lowered from 7.5, the ratio of operator affinity to nonspecific affinity is decreased, and more LexA repressor will bind to nontarget sites, resulting in decreased repression of the SOS genes. Data on the effects of pH on nonspecific binding are not available for pH values above 8.0, so it is unclear if target selectivity by intact LexA repressor drops off with increasing alkalinity.

The NMR structure of the N-terminal domain showed that the DNA-binding motif was a helix−turn−helix (Fogh et al., 1994). Computer docking simulations based on the NMR structure predicted possible direct contacts between LexA repressor and an operator half-site (Knegtel et al., 1995). The data in this study provide complementary information on how changes in environmental conditions affect the stability and specificity of operator binding by LexA repressor. We see that changes in anion and proton binding by LexA repressor appear to be linked to the overall operator-binding reaction, although our current data do not permit us to identify which of the multiple equilibria coupled to operator binding (e.g., any conformational changes in the protein, oligomerization of the protein, binding of the protein to operator DNA) requires these changes. Our observations do indicate, however, that changes in the intracellular physical environment may play a role in fine-tuning repression of the SOS system by LexA repressor.

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