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Ion Effects on the *lac* Repressor–Operator Equilibrium†

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ABSTRACT: The effects of ions on the interaction of *lac* repressor protein and operator DNA have been studied by the membrane filter technique. The equilibrium association constant was determined as a function of monovalent and divalent cation concentrations, anions, and pH. The binding of repressor and operator is extremely sensitive to the ionic environment. The dependence of the observed equilibrium constant on salt concentration is analyzed according to the binding theory of Record et al. [Record, M. T., Jr., Lohman, T. M., & deHaseth, P. L. (1976) *J. Mol. Biol. 107*, 145]. The number of ionic interactions in repressor–operator complex is deduced from the slopes of the linear log–log plots. About 11 ionic interactions are formed between repressor and DNA phosphates at pH 7.4 and about 9 ionic interactions at pH 8.0, in reasonable agreement with previous estimates. A favorable nonelectrostatic binding free energy of about 9–12 kcal/mol is estimated from the extrapolated equilibrium constants at the 1 M standard state. The values are in good accord with recent results for the salt-independent binding of repressor core and operator DNA. The effects of pH on the repressor–operator interaction are small, and probably result from titration of functional groups in the DNA-binding site of the protein. For monovalent salts, the equilibrium constant is slightly dependent on cation type and highly dependent on anion type. At constant salt concentration, the equilibrium constant decreases about 10 000-fold in the order $CH_3CO_2^- \geq F^- > Cl^- > Br^- > NO_3^- > SCN^- > I^-$. The wide range of accessible equilibrium constants provides a useful tool for in vitro studies of the repressor–operator interaction.

The interaction of *lac* repressor protein with DNA is influenced by ionic conditions. In the accompanying paper (Barkley, 1981), we examined the salt dependence of the kinetics of binding of the repressor and its operator site on a large DNA molecule. The unusual dependence of the association rate on cation concentration is consistent with mechanisms involving transitional binding of the protein to nonoperator DNA. Using Berg & Blomberg's (1978) theory for the sliding model, we deduced that diffusion of repressor along the DNA chain is about 4 times faster for MgDNA than for NaDNA. This paper reports an investigation of the effects of ions on the *lac* repressor–operator equilibrium. The affinity of repressor for operator is determined as a function of the concentration and type of salt and of pH, and the ion effects are interpreted according to the binding theory outlined below. The findings for the repressor–operator interaction are compared to published results for the repressor–DNA interaction.

Record and co-workers (Record et al., 1976, 1978; deHaseth

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et al., 1977) have treated ion effects on ligand–nucleic acid equilibria by incorporating Manning's polyelectrolyte theory in a multiple equilibria formulation. Because their analysis is based on thermodynamics, it applies both to site binding and to territorial binding of cationic ligands to polynucleotides. Association of a protein, having Z positive charges in its binding site, and a DNA molecule (e.g., eq 3) neutralizes Z phosphates and releases $Z\psi$ counterions from the DNA. In the presence of only monovalent salt, MX, the observed equilibrium association constant K_{obsd} varies with counterion concentration according to eq 1.

$$\frac{\partial \ln K_{obsd}}{\partial \ln [M^+]} = -Z\psi \quad (1)$$

In the absence of anion release from the protein, ψ is a constant and the log–log plot of K_{obsd} vs. $[M^+]$ is linear. Here, ψ is the number of counterions thermodynamically bound per phosphate: $\psi = \psi_c + \psi_s$, the sum of contributions from condensation (ψ_c) and from Debye–Hückel screening (ψ_s). Once $Z\psi$ is known from the slope of the log–log plot, the thermodynamic equilibrium constant $K_T°$ may be obtained from eq 2 [cf. eq

$$\ln K_{obsd} = \ln K_T° + Z\xi^{-1} \ln \gamma_{\pm}\delta - Z\psi \ln [M^+] \quad (2)$$

7.17 of Record et al. (1978)], where $\gamma_{\pm}$ is the mean activity coefficient of the ions and where, for double-helical DNA, $\psi = 0.88$, $\xi = 4.2$, and $\delta = 0.56$. This permits estimation of the nonelectrostatic component of the binding free energy for the protein–DNA interaction. The binding theory has been extended to include effects of divalent cations, anions, and pH on K_{obsd}. In the presence of only divalent salt, MX_2, expressions analogous to eq 1 and 2 apply, except that $\psi = 0.47$ for double-helical DNA in the limit of no anion release and $\delta = 0.80$. Competitive binding of anions to the Z positive charges in the binding site of the protein, or of divalent cations to the DNA, introduces additional terms in eq 1 and 2, which result in curvature of the log–log plot. The effects of pH on K_{obsd} have been analyzed for two limiting cases: (1) a titration curve, in which the number of ionic interactions depends on the pH, and (2) a requirement for protonation, in which the pKs of some functional groups on the protein shift upon complexation. The titration curve model predicts that the salt dependence of K_{obsd} be a function of pH and, conversely, that the pH dependence of K_{obsd} be a function of salt concentration. On the other hand, the requirement for protonation model predicts that effects of salt concentration and pH on K_{obsd} be essentially independent.

The binding of *lac* repressor protein and nonoperator DNA has been studied in considerable detail. deHaseth et al. (1977) and Revzin & von Hippel (1977) have determined the dependence of the equilibrium constant K_{obsd}^{RD} on ionic conditions and temperature and analyzed their results using the binding theory described above. The formation of repressor–DNA complex is accompanied by release of about 11 monovalent cations and uptake of about two protons. The affinity extrapolates to very low values at 1 M salt, indicating that the repressor–DNA interaction is predominantly electrostatic.

Materials and Methods

The standard (BB), phosphate (P-BB), and Tris[1] (T-BB) binding buffers are described in the preceding paper (Barkley, 1981). The purified *Escherichia coli lac* repressor and λp*lac*5 DNA used in membrane filter experiments are also described there.

The observed reaction of repressor (R) and operator (O) is

$$R + O \overset{K_{obsd}}{\rightleftharpoons} RO \quad (3)$$

where

$$K_{obsd} = \frac{[RO]}{[R][O]} \quad (4)$$

The equilibrium constant K_{obsd} can be determined from the ratio of the rate constants for the association and dissociation reactions, if the same steps are rate limiting under the conditions of the equilibrium and kinetic measurements (Riggs et al., 1970a). At higher salt concentrations, this situation obtains. Since kinetic data are more precise than equilibrium data (Riggs et al., 1970a), equilibrium association constants were calculated from $K_{obsd} = k_a/k_d$, where k_a is the association rate constant and k_d is the dissociation rate constant. The rate constants were measured as before (Barkley, 1981), and the equilibrium constant was computed from the mean values of the rate constants ± propagated standard deviations. At lower salt concentrations, the equilibrium constant cannot be determined from kinetic data as discussed under Results. For very high-affinity binding, the specific activity of the labeled DNA and the long equilibration times render the equilibrium binding measurements of low accuracy. Thus, we have not determined equilibrium constants at lower salt concentrations.

For low-affinity binding, equilibrium association constants were measured in equilibrium binding experiments as described by Riggs et al. (1970b), using constant operator concentrations [$(0.5–500) \times 10^{-12}$ M] and variable repressor concentrations. Equilibrium constants were obtained by fitting the data to binding curves calculated from eq 4 and are reported as mean values ± standard deviations of three to six experiments. At higher salt concentrations, equilibrium constants determined from kinetic data and from equilibrium data agreed within experimental error.

Results

Salt Dependence. The equilibrium constant for the binding of *lac* repressor protein and operator DNA was determined as a function of cation concentration for various salts and buffers: NaCl in phosphate buffer, pH 7.4; NaCl in Tris buffer, pH 8.0; $NaCH_3CO_2$ in Tris buffer, pH 8.0; $MgCl_2$ in Tris buffer, pH 8.0. Because small changes in K_{obsd} are readily detected from kinetic data, we have computed $K_{obsd} = k_a/k_d$ from the mean values of the rate constants given in the preceding paper [cf. Figures 1–3, 5, and 6 of Barkley (1981)]. The results presented there indicate that the kinetics of repressor–operator binding can be described by the reaction of eq 3 at higher salt concentrations. However, at intermediate and lower salt concentrations, the association kinetics change from bimolecular to apparent first order; the bimolecular rate constant k_a becomes inversely proportional to the concentration of operator DNA at lower salt concentrations. Berg & Blomberg's (1978) theory for the salt dependence of the association kinetics also predicts that the rate constant k_a depends inversely on operator concentration at intermediate and lower salt concentrations. Thus, the equilibrium association constant K_{obsd} can be determined from the ratio of the rate constants k_a/k_d only at higher salt concentrations. The salt dependence of K_{obsd} at higher salt concentrations is portrayed according to the binding theory (Record et al., 1976, 1978) in Figures

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; IPTG, isopropyl β-D-thiogalactoside; ANS, 8-anilino-1-naphthalenesulfonate; bis-ANS, bis[5,5′-(8-anilino-1-naphthalenesulfonate)].

Table I: Equilibrium Parameters

conditions[a]	no. of counterions released,[b] $Z\psi$	ln K_{obsd} (1 M)[c]	no. of ionic interactions,[d] Z	ln $K_T°$ [e]	nonelectrostatic component of ln $K_T°$ [f]
NaCl, P-BB, pH 7.4	9.3 ± 0.4	11.9 ± 0.7	10.6 ± 0.5	14.1 ± 0.7	16.3
NaCl, T-BB, pH 8.0	7.7 ± 0.3	14.1 ± 0.5	8.8 ± 0.3	15.9 ± 0.5	17.7
$NaCH_3CO_2$, T-BB, pH 8.0	7.5 ± 0.3	17.9 ± 0.5	8.5 ± 0.3	19.7 ± 0.5	21.4
$MgCl_2$, T-BB, pH 8.0	4.1 ± 0.2	11.7 ± 0.9	8.7 ± 0.4	12.9 ± 0.5	14.6

[a] Measurements at 20 °C. [b] The negative least-squares slope ± SD of plots in Figures 1 and 2. [c] The least-squares intercept ± SD of plots in Figures 1 and 2. [d] Computed from the values in column 2, with $\psi_{M^+} = 0.88$ and $\psi_{M^{2+}} = 0.47$. [e] Computed according to eq 2 as described in the text. [f] Corrected for the contribution of Z lysine-like ionic interactions as described in the text. Nonelectrostatic component of ln $K_T° = \ln K_T° + 0.2Z$.

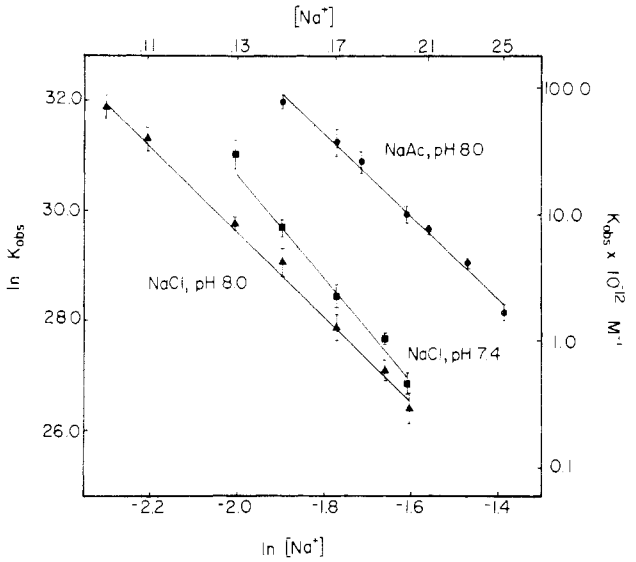

FIGURE 1: Dependence of the equilibrium constant of *lac* repressor protein and λp*lac* DNA on cation concentration for NaCl, pH 7.4 and pH 8.0, and for $NaCH_3CO_2$, pH 8.0. Symbols are experimental values ± SD indicated by error bars: (■) NaCl, P-BB, pH 7.4; (▲) NaCl, T-BB, pH 8.0; (●) $NaCH_3CO_2$, T-BB, pH 8.0. Lines drawn are least-squares fit through the points.

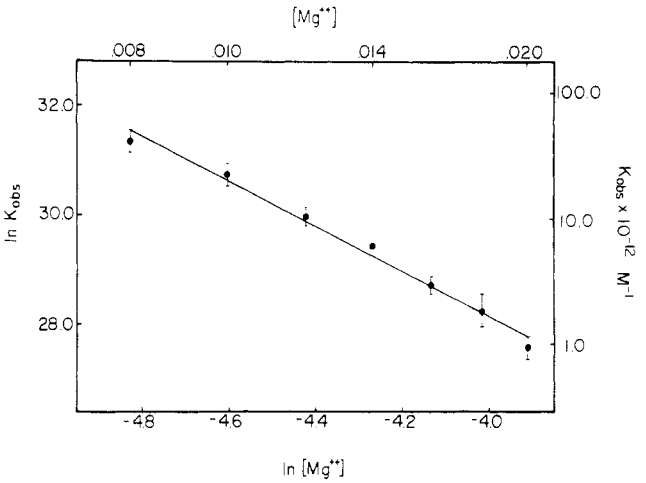

FIGURE 2: Dependence of the equilibrium constant of *lac* repressor protein and λp*lac* DNA on cation concentration for $MgCl_2$, T-BB, pH 8.0. Symbols are experimental values ± SD indicated by error bars. Line drawn is least-squares fit through the points.

1 and 2. In every case, the log–log plots of the equilibrium constant K_{obsd} vs. cation concentration are linear, within experimental error. The absence of curvature suggests that anions are not released from the DNA-binding site of the protein upon formation of repressor–operator complex. However, the range of salt concentrations investigated for each condition is too narrow to secure this conclusion.

Comparison of the results for Na^+ in Figure 1 and for $MgCl_2$ in Figure 2 shows that Mg^{2+} competes more effectively than Na^+ with *lac* repressor for binding to operator DNA. That is, the cation concentration required to achieve a particular value of K_{obsd} (e.g., 1×10^{13} M^{-1}) is much lower for $MgCl_2$ (0.012 M) than for NaCl (0.13 M). Moreover, effects of anion release should be reduced in the presence of $MgCl_2$, since the anion concentration is also lower (deHaseth et al., 1977). As expected from the relative magnitudes of ψ for mono- and divalent cations ($\psi_{M^+}/\psi_{M^{2+}} = 1.9$) in eq 1, the observed salt dependence of the equilibrium constant is greater for Na^+ than for Mg^{2+}. The finding of linear log–log plots for Na^+ and for Mg^{2+}, with the predicted 1.9-fold difference in slope at pH 8.0 (Table I, column 2), supports the interpretation that anion release is not an important factor.

(*a*) *Number of Ionic Interactions in RO Complex Depends on pH.* In the absence of anion release, the slopes of the log–log plots in Figures 1 and 2 give the number of counterions released from DNA upon formation of repressor–operator complex. In Figure 1, the slope $\partial \ln K_{obsd}/\partial \ln [Na^+]$ is steeper at pH 7.4 than at pH 8.0, implying that more counterions are released at the lower pH. The negative least-squares slopes, $Z\psi$, for various conditions are given in column 2 of Table I. About nine monovalent counterions are released at pH 7.4, compared to about eight at pH 8.0. Also, about four divalent counterions are released at pH 8.0, half the number of monovalent counterions. This is the ratio one would expect if the salt dependence were governed by cation release, since a divalent cation neutralizes twice as many DNA phosphates as a monovalent cation.

The number of ionic interactions, Z, formed between the repressor and phosphate charges on the operator is computed from the experimental value of $Z\psi$ with the appropriate value of ψ, and tabulated in column 4 of Table I. Z is about 11 at pH 7.4 and about 9 at pH 8.0. The finding of linear log–log relationships between K_{obsd} and cation concentration at the two pHs, but a decreased value of Z at the higher pH, is consistent with the titration curve model [cf. eq 7.30 of Record et al. (1978)]. This suggests that there are functional groups in the DNA-binding site of the protein which are susceptible to deprotonation in the pH range investigated.

(*b*) *Thermodynamic Equilibrium Constant Depends on the Anion.* The thermodynamic equilibrium constant, $K_T°$, may be deduced from the value of K_{obsd} at the 1 M standard state. The least-squares intercepts of the log–log plots in Figures 1 and 2 at ln $[Na^+] = 0$ or ln $[Mg^{2+}] = 0$ yield the values of ln K_{obsd} (1 M) given in column 3 of Table I. From the experimental values of Z and ln K_{obsd} (1 M), ln $K_T°$ is calculated according to eq 2 with the appropriate value of δ and with an average value of $\gamma_\pm$ for the salt concentration range investigated (Robinson & Stokes, 1965). The values of ln $K_T°$ for various conditions are given in column 5 of Table I. The titration curve model predicts that ln $K_T°$ be independent of

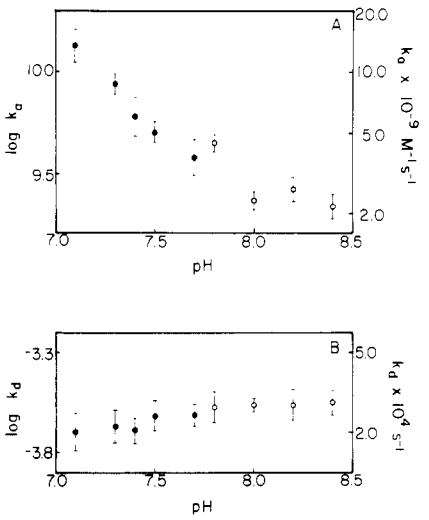

FIGURE 3: Dependence of the rate constants of *lac* repressor protein and λp*lac* DNA on pH for NaCl, $[Na^+]$ = 0.13 M. Symbols are experimental values ± SD indicated by error bars: (●) P-BB, pH 7.1–7.7; (○) T-BB, pH 7.8–8.4. (A) Association rate constant. (B) Dissociation rate constant.

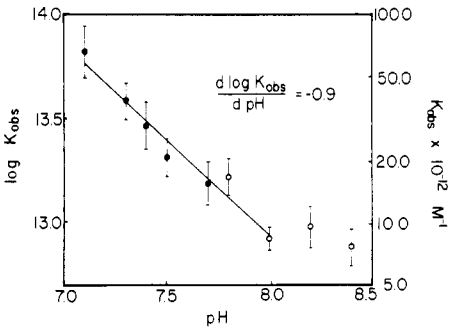

FIGURE 4: Dependence of the equilibrium constant of *lac* repressor protein and λp*lac* DNA on pH for NaCl, $[Na^+]$ = 0.13 M. Symbols represent values of K_{obsd} calculated from values of k_a and k_d in Figure 3 ± SD indicated by error bars: (●) P-BB, pH 7.1–7.7; (○) T-BB, pH 7.8–8.4. Line drawn is the least-squares fit through the points for pH ≤8.0.

pH [cf. eq 7.29 of Record et al. (1978)]. The values of ln $K_T°$ for NaCl at pH 7.4 and 8.0 differ by 12%, slightly more than the propagated experimental error (3–5%). This could reflect differential anion release at the two pHs, inaccuracy of the long extrapolations, or deviation from the simple titration curve model.

A substantial anion effect on the equilibrium constant is visible in Figure 1. The plot for $NaCH_3CO_2$ is displaced toward higher cation concentrations compared to the plots for NaCl. At the same ion concentration and pH, K_{obsd} is about 30 times larger in acetate than in chloride. A comparable difference between K_{obsd}^{RD} in acetate and in chloride for the repressor–DNA interaction was found by deHaseth et al. (1977). They offered two possible interpretations, which were consistent with their data: the release of one Cl^- from the DNA-binding site of the repressor or a conformational change in the repressor induced by binding of $CH_3CO_2^-$ elsewhere on the protein. In Table I, the negative slopes ($Z\psi$), which count the total number of ions released upon formation of repressor–operator complex, differ by only 3% in acetate and in chloride at pH 8.0. As this difference is within experimental error (4%), we conclude that there are no major differences in the number of anions released, if any, in the two conditions. The *lac* repressor is an allosteric protein, so it is possible that anions affect K_{obsd} via conformational alterations in the repressor.

For $NaCH_3CO_2$, ln $K_T°$ is about 20, compared to an average value of 15.0 ± 0.9 for NaCl and about 13 for $MgCl_2$. Since the thermodynamic equilibrium constant measures nonionic contributions to the binding free energy, the difference between ln $K_T°$ in acetate and in chloride is consistent with a conformational change in the repressor. In those cases where chloride is the anion, ln $K_T°$ is expected to be greater for Na^+ than for Mg^{2+} [cf. eq 21 of deHaseth et al. (1977)]. The observed difference between ln $K_T°$ in NaCl and in $MgCl_2$ of about 2–3 ln units is quite close to the predicted difference of about 3–4 ln units (estimated from the data for the Mg^{2+}–DNA interaction in Tris buffer; Record et al., 1977).

Ion Type. The analyses of the dependence of k_a, k_d, and K_{obsd} on cation concentration presented in these two papers [see Barkley (1981)] reveal that the kinetic and equilibrium binding parameters are affected by pH and by the type of cation and anion. In order to examine the effects of ion type on the *lac* repressor–operator interaction, we performed additional experiments holding the monovalent cation concentration constant at $[M^+]$ = 0.13 M.

(*a*) *Effects of pH Are Small.* The association and dissociation rates were measured over the pH range 7.1–8.4 for NaCl in phosphate or Tris buffer. The pH dependence of the rate constants is shown in Figure 3. There is no apparent buffer effect on the repressor–operator interaction at this salt concentration. The association rate constant k_a decreases about 6-fold from pH 7.1 to 8.0 and becomes independent of pH above 8.0, within experimental error. The dissociation rate constant k_d does not depend significantly on pH, increasing at most by about 40% from pH 7.1 to 8.4, if at all. Riggs et al. (1970a) found a similar pH dependence in standard buffer (BB, pH 7–9).

The pH dependence of the equilibrium constant K_{obsd} is depicted in Figure 4. From pH 7.1 to 8.0, log K_{obsd} decreases linearly with pH, with a least-squares slope d log K_{obsd}/(d pH) = −0.9 ± 0.1. Above pH 8.0, K_{obsd} is constant within experimental error. Although we have not explicitly investigated the pH dependence at other salt concentrations, we estimate d log K_{obsd}/(d pH) ~ −0.3 at $[Na^+]$ = 0.20 M by using values of K_{obsd} for NaCl at pH 7.4 and 8.0 (Figure 1). From the data of Riggs et al., we estimate d log K_{obsd}/(d pH) ~ −0.6 for the pH range 7.4–8.2 [BB has an effective cation concentration equivalent to $[Na^+]$ ~ 0.12 M; cf. eq 5 of Record et al. (1977)]. These weak pH dependences, plus the lack of dependence at alkaline pH, support the titration curve model. This model predicts a dependence of d log K_{obsd}/(d pH) on $[M^+]$, which decreases in magnitude with increasing salt concentration and becomes negligible at high salt. At sufficiently alkaline pH, the susceptible groups in the DNA-binding site of the protein are deprotonated, so K_{obsd} would be independent of pH.

In contrast, deHaseth et al. (1977) reported a larger effect of pH on the repressor–DNA interaction. For [NaCl] = 0.13 M over the pH range 7.7–8.4, log K_{obsd}^{RD} decreases linearly with pH, with a slope of −2.1. They interpreted the pH effect as a requirement for protonation and deduced that the formation of repressor–DNA complex is accompanied by uptake of two protons by the protein. This conclusion is consistent with their findings that the number of ionic interactions in repressor–DNA complex is the same at pH 7.4 and 8.0 within experimental error and that the reaction has a sizable, negative enthalpy at alkaline pH.

The effects of pH on the repressor–operator interaction are perplexing, when viewed in light of these results for the repressor–DNA interaction. The pH dependence of the equi-

Table II: Cation Effects

cation[a]	$k_a \times 10^{-9}$ (M^{-1} s^{-1})	$k_d \times 10^4$ (s^{-1})	$K_{obsd} \times 10^{-12}$ (M^{-1})[b]	$K_{obsd}^{MCl}/K_{obsd}^{NaCl}$
Cs^+	6.0 ± 0.3	3.5 ± 0.7[c]	17 ± 4 15 ± 6[d]	1.9
K^+	4.1 ± 0.6	3.6 ± 0.3	11 ± 2	1.4
Na^+	2.3 ± 0.2	2.7 ± 0.2	8 ± 1	1.0
NH_4^+	2.3 ± 0.3	3.4 ± 0.2	7 ± 1	0.81
Li^+	1.15 ± 0.04	11 ± 3	1.0 ± 0.3	0.12

[a] Measurements in T-BB, [MCl] = 0.13 M at 20 °C. [b] Calculated from $K_{obsd} = k_a/k_d$ ± SD of propagated errors. [c] High backgrounds (10–20%) were observed in the dissociation rate experiments for CsCl purchased from several sources. [d] Determined from equilibrium binding experiments.

librium constant K_{obsd} arises primarily from k_a, since k_d is virtually insensitive to pH. If, as in the case of the salt dependence, the association rate depends on pH via the affinity of repressor for nonoperator DNA, then k_a and K_{obsd} should continue to decrease at more alkaline pH. The dependence of K_{obsd}^{RD} on pH from 7.1 to 7.7 at $[Na^+]$ = 0.13 M has not been reported, so we have not attempted to fit Berg & Blomberg's (1978) theory to the data for the association rate constant k_a in Figure 3.

(*b*) *Effects of Monovalent Cations Are Small.* The binding parameters for various monovalent chloride salts in Tris buffer at pH 8.0 are given in Table II. The equilibrium constant K_{obsd} decreases about 16-fold in the order $Cs^+ \gtrsim K^+ \gtrsim Na^+ \gtrsim NH_4^+ > Li^+$. The association rate constant k_a decreases about 5-fold in the same order, and the dissociation rate constant k_d is insensitive to cation type,[2] with the exception of Li^+. The effect of M^+ relative to Na^+ on the *lac* repressor–operator equilibrium is presented in the last column of Table II. For Cs^+, K^+, Na^+, and NH_4^+, the values of K_{obsd} differ by at most a factor of 2; for Li^+, the value of K_{obsd} is about a factor of 10 less than the values for the other cations. These relatively small effects on the binding parameters could arise from cation effects on the DNA or on the repressor, or from a combination of effects on the protein and DNA.

Because of problems associated with the membrane filter assay, we have not been able to determine the effect of divalent cation type. Most divalent cations in T-BB caused the ^{32}P-labeled λp*lac*5 DNA to bind quantitatively to the filters in the absence of repressor. The only exceptions were Ca^{2+} and Ba^{2+}. We performed some preliminary experiments in T-BB, $[CaCl_2]$ = 0.01 M or $[BaCl_2]$ = 0.01 M, with results comparable to those for $MgCl_2$. However, subsequent experiments yielded erratic results.

(*c*) *Effects of Monovalent Anions Are Large and Variable.* In contrast to the small effects of monovalent cations, monovalent anions have enormous effects on the *lac* repressor–operator interaction. The binding parameters for the sodium salts of various anions in Tris buffer at pH 8.0 are given in Table III. The equilibrium constant K_{obsd} decreases about 10 000-fold in the order $CH_3CO_2^- \gtrsim HCO_2^- \gtrsim CH_3CH_2CO_2^- \gtrsim F^- > HPO_4^{2-} > SO_4^{2-} \gtrsim$ citrate$^{3-} \gtrsim Cl^- > Br^- > NO_3^- > SCN^- \gtrsim I^-$. Both the association and dissociation rate constants are sensitive to anion type, with k_a decreasing and k_d increasing about 40-fold from $CH_3CO_2^-$ to NO_3^-. The relative effect of a given anion compared to chloride on the repressor–operator equilibrium is presented in the last column of Table III. The anions can be grouped as follows: (1) monovalent anions (fluoride, chloride, bromide, nitrate, thiocyanate, and iodide) which approximately follow the lyotropic series; (2) carboxylate anions (acetate, formate, and propionate) which increase K_{obsd} almost 20-fold compared to chloride; and (3) multivalent anions (phosphate, sulfate, and citrate) which have about the same effect as chloride. Because the effects of anions are large and the standard buffer contains two types of anions (acetate and chloride), we also included three pairs of anions: $CH_3CO_2^-/Cl^-$, Cl^-/NO_3^-, and Cl^-/I^-. The binding parameters for equimolar mixtures of these pairs are given in Table III. Two pairs, $CH_3CO_2^-/Cl^-$ and Cl^-/NO_3^-, act competitively, resulting in values of K_{obsd} intermediate to the values obtained in the presence of either anion alone. However, the Cl^-/I^- pair acts noncompetitively, resulting in a value of K_{obsd} lower than the values obtained in chloride or in iodide. This is further illustrated by the experiment in Figure 5. Here, constant amounts of repressor protein and operator DNA were equilibrated in binding buffers containing different proportions of Cl^- and I^-. The ordinate (% input) is proportional to the concentration of repressor–operator complex in the solution, which is governed by the value of K_{obsd} under the given ionic conditions. Similar experiments for $CH_3CO_2^-/Cl^-$ and Cl^-/NO_3^- (data not shown) gave essentially straight lines between the two ordinates. The results for Cl^-/I^- suggest that there is more than one anion binding site on the repressor.

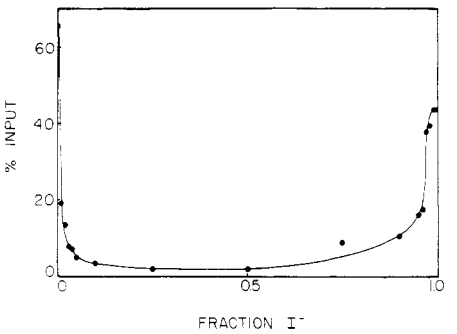

FIGURE 5: Effects of varying proportions of $[Cl^-]/[I^-]$ on the repressor–operator equilibrium. Constant amounts of *lac* repressor protein and λp*lac* DNA were equilibrated in T-BB containing different proportions of Cl^- and I^- at 0.13 M Na^+. The ordinate (% input) is proportional to the concentration of repressor–operator complex in solution. At the concentrations used in this experiment ($R = O = 1.2 \times 10^{-10}$ M), binding is stoichiometric in the presence of Cl^- alone but not in the presence of I^- alone.

As far as we can tell, the anion effects described above do not result from anomalies in the filter assay or from irreversible changes in the repressor protein. Titration experiments in binding buffers containing different anions yielded the expected values for the active repressor concentration. Moreover, incubation of the repressor with different anions or pairs of anions for 30 min at 4 °C, prior to dilution and assay in chloride, gave binding parameters characteristic of chloride. For binding buffers containing I^-, the results were independent of the absence or presence of trace amounts of I_3^- (determined by absorbance at 286 nm). However, we noted that the equilibration times required with the Cl^-/I^- pair were slightly longer.

The effects of anions on the repressor–operator interaction probably arise from anion binding to the protein, either at the DNA-binding site or at a separate site. In principle, the effects of multivalent anions could provide evidence for anion binding at the DNA-binding site of the repressor [cf. eq 10 of deHaseth

[2] We had previously reported that $k_d = (1.8 \pm 0.2) \times 10^{-4}$ s^{-1} in BB, 0.04 M KCl without $Mg(CH_3CO_2)_2$ [cf. Table II of Barkley et al. (1975)]. The data in these two papers [see Barkley (1981)] indicate that the value should have been about 10-fold lower. This error plus an invalid assumption, that k_a is independent of $[Mg^{2+}]$, negates the conclusion that magnesium exerts specific effects on the induction phenomenon.

Table III: Anion Effects

anion[a]	$k_a \times 10^{-9}$ (M^{-1} s^{-1})	$k_d \times 10^4$ (s^{-1})	K_{obsd} (M^{-1})[b]	$K_{obsd}^{Na_nX}/K_{obsd}^{NaCl}$
$CH_3CO_2^-$	10 ± 1	0.64 ± 0.05	(1.6 ± 0.3) × 10^{14}	19
HCO_2^-	9 ± 2	0.62 ± 0.02	(1.5 ± 0.3) × 10^{14}	17
$CH_3CH_2CO_2^-$	9.5 ± 0.3	0.71 ± 0.05	(1.34 ± 0.04) × 10^{14}	16
F^-	9 ± 2	0.74 ± 0.04	(1.3 ± 0.3) × 10^{14}	15
HPO_4^{2-}	4.6 ± 0.2	2.0 ± 0.2	(2.3 ± 0.2) × 10^{13}	2.7
SO_4^{2-}	3.0 ± 0.3	2.7 ± 0.1	(1.1 ± 0.1) × 10^{13}	1.3 (0.24[d])
$citrate^{3-}$	2.8 ± 0.4	2.9 ± 0.5	(9 ± 2) × 10^{12}	1.1
Cl^-	2.3 ± 0.2	2.7 ± 0.2	(8.± 1) × 10^{12}	1.0
Br^-	1.3 ± 0.2	14.7 ± 0.7	(9 ± 1) × 10^{11}	0.11
NO_3^-	0.27 ± 0.03	23 ± 4	(1.2 ± 0.3) × 10^{11}	0.018
			(1.9 ± 0.7) × 10^{11} [c]	
SCN^-			(1.8 ± 0.5) × 10^{10} [c]	0.0021
I^-			(1.3 ± 0.6) × 10^{10} [c]	0.0015
$CH_3CO_2^-/Cl^-$	3.1 ± 0.5	1.7 ± 0.3	(1.9 ± 0.4) × 10^{13}	2.2
Cl^-/NO_3^-	0.49 ± 0.05	17 ± 2	(2.9 ± 0.5) × 10^{11}	0.048
			(5 ± 2) × 10^{11} [c]	
Cl^-/I^-			~1 × 10^9 [c]	~0.0001

[a] Measurements in T-BB, [Na^+] = 0.13 M at 20 °C. Pairs of anions are present in equimolar amounts. [b] Calculated from $K_{obsd} = k_a/k_d$ ± SD of propagated errors. [c] Determined from equilibrium binding experiments. [d] Corrected for incomplete dissociation of $NaSO_4^-$ (Robinson & Stokes, 1965). Ratio computed relative to the value of K_{obsd}^{NaCl} at [NaCl] = 0.107 M, pH 8.0, estimated from the least-squares lines in Figure 1.

et al. (1977)]. Because the multivalent anions are present in lower concentrations than the monovalent anions at [Na^+] = 0.13 M, their ability to compete with DNA phosphates would be reduced. With the assumption that the multivalent anions bind with about the same affinity as chloride, given the comparable effects on K_{obsd}, the values of K_{obsd} should increase with increasing anion valence (i.e., decreasing anion concentration). The data in Table III do not fit this simple scheme. For HPO_4^{2-}, the value of K_{obsd} is about a factor of three larger than the value for Cl^-, whereas for SO_4^{2-}, the value of K_{obsd} is actually a factor of four less after correction for incomplete dissociation of $NaSO_4^-$. For $citrate^{3-}$, the value of K_{obsd} is the same as for Cl^- within experimental error.

Discussion

Ion Dependence of the Equilibrium Constant. The equilibrium association constant K_{obsd} is a sensitive function of ionic environment, which depends on the concentration and type of ions and on the pH. Analyzing the dependence of K_{obsd} on cation concentration according to the binding theory of Record and co-workers (Record et al., 1976, 1978), we find that about 8–9 monovalent or about 4 divalent counterions are released from DNA upon binding of *lac* repressor and operator and that about 9–11 ionic interactions are formed between the repressor and phosphate groups on DNA in repressor–operator complex. Our results are consistent with the predictions of Record et al. (1977), which were obtained by correcting the data of Riggs et al. (1970b) for the presence of Mg^{2+}. Figure 6 shows the corrected and experimental values of K_{obsd}. The broken and solid lines denote corrected equilibrium constants obtained from equilibrium (Record et al., 1977) and kinetic (Lohman et al., 1978) data, respectively; the points represent equilibrium constants computed from kinetic data [cf. Figure 7 of Barkley (1981)], and the dotted line denotes the least-squares line through the data at higher salt concentrations from Figure 1. The agreement between the corrected and experimental values of K_{obsd} is quite good. The slopes differ by slightly more than the estimated errors, giving $Z = 8 \pm 1$ for the corrected data and $Z = 10.6 \pm 0.5$ for the experimental data. Adjusting for the small K^+ ion effect (Table II) would raise the experimental values by 0.13 log unit, and adjusting for a difference in the repressor (Riggs et al. reported $K_{obsd} = 1 \times 10^{13}$ M^{-1} under standard conditions whereas we obtain $K_{obsd} = 3 \times 10^{13}$ M^{-1}; Barkley, 1981) would lower them by

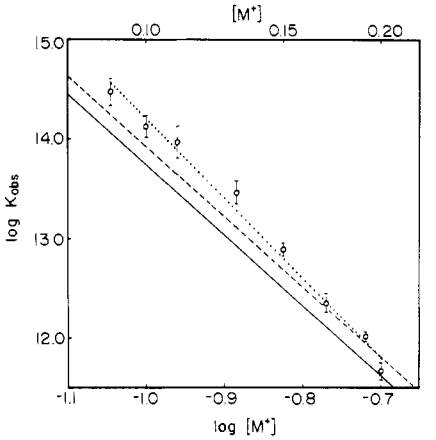

FIGURE 6: Dependence of the equilibrium constant on monovalent cation concentration. Points represent values of K_{obsd} calculated from values of k_a and k_d given in the preceding paper [cf. Figure 7 of Barkley (1981)]. The dotted line is the least-squares fit through the points. The broken line is calculated according to log K_{obsd} = −7.04 log [K^+] + 6.9 (Record et al., 1977). The solid line is calculated according to log K_{obsd} = −7.04 log [K^+] + 6.7 (Lohman et al., 1978).

−0.48 log unit. Compensating for both K^+ and the repressor would lower the experimental values by −0.35 log unit. With these changes, most of the points fall within the range of the corrected values. This comparison, as well as the one in the preceding paper (Barkley, 1981), shows that the primary effect of Mg^{2+} on the repressor–operator interaction in the presence of both mono- and divalent cations arises from competition between Mg^{2+} and the repressor for binding to DNA. As described under Results, we find that the number of ionic interactions depends on pH, with Z being about 11 at pH 7.4 and about 9 at pH 8.0. The value at pH 7.4 is based on a single determination in phosphate buffer, whereas the value at pH 8.0 is based on three determinations in Tris buffer which agreed within experimental error. It is possible that the apparent pH dependence arises from a systematic error, though we have no evidence for buffer effects. However, if this were the case, then the mean value of $Z = 9 \pm 1$ would agree with the predicted value of $Z = 8 \pm 1$ of Record et al., within experimental error. Recently, O'Gorman et al. (1980) have determined the dependence of K_{obsd} on NaCl concentration in Tris buffer at pH 7.5 from equilibrium binding experiments and estimated $Z = 5 \pm 1.5$. This value deviates from the above

values by more than the estimated errors. The discrepancy is probably due to systematic errors, since the values of K_{obsd} reported by these authors are substantially lower than the values reported here and somewhat lower than the values reported by Riggs et al. (1970a,b) in the presence of competition by 0.01 M Mg^{2+}.

We have also estimated the nonelectrostatic component of the free energy of interaction of *lac* repressor and operator. In a model study, Lohman et al. (1980) reported that ln K_{obsd} (1 M) = -2.1 ± 0.7 for the pentalysine–DNA interaction, giving ln $K_T° = -1.0$. This reflects a contribution of all other factors besides counterion release to the thermodynamic equilibrium constant of about −0.2 ln unit per cationic group. Assuming Z lysine-like ionic interactions in repressor–operator complex, the nonelectrostatic component of ln $K_T°$ for various conditions is computed in the last column of Table I. The value of about 17 for NaCl is quite close to the value of about 19 for KCl predicted by Record et al. (1978) from the data of Riggs et al. (1970a,b). At 20 °C, the favorable nonelectrostatic component of the binding free energy amounts to about 9–10 kcal/mol for NaCl and $MgCl_2$ and about 12 kcal/mol for $NaCH_3CO_2$. Matthews (1979) has demonstrated that *lac* repressor core protein, which is missing the amino-terminal 59 residues of the polypeptide, binds to operator DNA, although it does not bind detectably to nonoperator DNA. The operator DNA binding of the repressor core is sensitive to inducer and independent of salt concentration ($Z < 0.02$; O'Gorman et al., 1980). The equilibrium constant for the repressor core–operator interaction is $K \simeq 1 \times 10^7$ M^{-1} in the standard buffer and $K = (2–3) \times 10^7$ M^{-1} for NaCl in Tris buffer at pH 7.5, corresponding to a favorable binding free energy at 20 °C of about 9–10 kcal/mol. The amino-terminal fragment binds specifically to the *lac* operator (Ogata & Gilbert, 1978). The fragment also binds to nonoperator DNA, and the binding depends on ionic conditions (Jovin et al., 1977). The free energies of the repressor core–operator interaction and the nonelectrostatic component of the repressor–operator interaction are in good accord. This finding supports models of the repressor–operator interaction, such as the one proposed recently by Matthews and co-workers (O'Gorman et al., 1980; Dunaway et al., 1980), in which the amino-terminal region of the protein forms primarily ionic interactions and the core region primarily nonionic interactions with the operator site.

The effects of pH on the *lac* repressor–operator interaction apparently result from titration of functional groups in the DNA-binding site of the protein. Similar pH effects have been observed for the pentalysine–DNA interaction (Lohman et al., 1980) and for the interactions of *E. coli* RNA polymerase core and holoenzymes and DNA (deHaseth et al., 1978). The *lac* repressor protein has two groups located in the amino-terminal region which would be susceptible to deprotonation around neutral pH: the terminal amino group and the histidine residue at position 29. The pH dependence of the repressor core–operator interaction has not been reported. However, the tentative assignment above, together with the fact that pH effects on K_{obsd} come from the association reaction (via nonoperator DNA binding), suggests that repressor core binding would be independent of pH.

The relatively small effects of monovalent cation type on the equilibrium constant K_{obsd} argue that cations have an insignificant effect, if any, on the *lac* repressor. Except for Li^+, the effects of monovalent cations on proteins are generally small [see von Hippel & Schleich (1969)]. Our findings for the repressor–operator interaction raise the possibility of a cation effect on the protein, since Li^+ is the only monovalent cation with an appreciable effect. However, the effects of Mg^{2+} on K_{obsd} are adequately explained by competitive binding to DNA. The effect of cations on the other biological activity of the *lac* repressor, binding of inducers and anti-inducers, has not been investigated. Two measurements of inducer IPTG binding under comparable conditions (0.2 M MCl, pH 7.5–7.6, 25 °C) give equilibrium constants of $\sim 4.0 \times 10^5$ M^{-1} for NaCl (Friedman et al., 1977) and $\sim 4.7 \times 10^5$ M^{-1} for KCl (Butler et al., 1977), also exhibiting no significant cation effect.

The effects of cation type on the physical properties of DNA have been extensively studied. For example, Anderson & Bauer (1978) deduced that the duplex rotation angle of DNA increases with cation type in the order $Na^+ < K^+ < Li^+ < Rb^+ < Cs^+ < NH_4^+$ from the degree of supercoiling of bacteriophage PM-2 DNA. Hanlon and co-workers (Wolf & Hanlon, 1975; Chan et al., 1979) found the same order from circular dichroism studies of calf thymus DNA. Recently, Bleam et al. (1980) reported that the relative binding affinities of monovalent cations increase in the order $Na^+ < Li^+ < K^+ < Cs^+ < NH_4^+$ on the basis of ^{23}Na NMR line-width measurements. Since binding of *lac* repressor and operator DNA is accompanied by a small amount of unwinding of the DNA duplex (about 90° in standard buffer; Wang et al., 1974), we might expect that K_{obsd} would decrease with increasing duplex rotation angle. Alternatively, the effects of cations on K_{obsd} could result from competition between the repressor and DNA counterions; at the same salt concentration, K_{obsd} would decrease with increasing affinity of the counterion for DNA. Neither of the above orderings correlates with that for K_{obsd}. However, this is not surprising as the differences in duplex rotation angle or in binding affinities of monovalent cations are slight.

The origin and significance of the marked effects of monovalent anions on the *lac* repressor–operator equilibrium are not known. We will mention briefly some possible explanations of our results. For the two anions acetate and chloride, which have been examined in most detail, the log–log plots of K_{obsd} vs. cation concentration show no evidence of curvature or of differences in slope beyond that predicted for mono- and divalent cations. According to the binding theory, this could mean either that anion release from the repressor is negligible (cf. eq 1) or that the same number of anions are released in acetate and in chloride (cf. eq 5). If no anions are released upon binding to DNA, then anions must exert an indirect effect on the equilibrium constant K_{obsd}, presumably by causing conformational changes in the repressor. A range of anion effects could arise from shifts in the conformational equilibrium of a two-state model. Alternatively, they could result from a series of discrete conformations. In both cases, the values of the thermodynamic equilibrium constant $K_T°$ (estimated by extrapolation of the log–log plots) would reflect nonelectrostatic contributions to the binding free energy of the various conformational states. Conformational changes in the *lac* repressor protein upon binding of inducers and anti-inducers are well-documented phenomena [see Miller & Reznikoff (1978)] which play an important role in regulation of the *lac* operon. Inducers decrease the affinity of repressor for operator up to 1000-fold, whereas anti-inducers increase the affinity by about 5-fold (Barkley et al., 1975). However, these ligands do not affect the affinity of repressor for nonoperator DNA. This contrasts with the behavior of anions, which have roughly the same effect on operator and nonoperator DNA binding in the case of acetate and chloride. On the other hand, if anions are released from the repressor, eq 1 becomes [cf. eq 12 of deHaseth et al. (1977)]

$$\frac{\partial \ln K_{obsd}}{\partial \ln [M^+]} = Z\psi + a \quad (5)$$

where a is the number of anions released from the protein. If $K_x[X^-] \gg 1$, where K_x is the intrinsic binding constant for the anion, then a is a constant and the log–log plot will be linear. In this situation, the intercept at 1 M salt, ln K_{obsd} (1 M), includes the anion participation. The thermodynamic equilibrium constant $K_T°$ cannot be determined from the intercept without independent information about anion binding. Moreover, the value of Z deduced from the slope of the log–log plot is a maximum estimate of the number of ionic interactions in the protein–DNA complex. For example [using the complete form of eq 5 given by deHaseth et al. (1977)], one anion binding site on the repressor, having $K_{Ac} \sim 25$ M^{-1}, $K_{Cl} \sim 10^3$ M^{-1}, and $K_I \sim 10^7$ M^{-1}, would explain the values of K_{obsd} for Na^+ but overestimate the values for Mg^{2+} by about a factor of seven. Two identical anion binding sites with $K_{Ac} \sim 25$ M^{-1}, $K_{Cl} \sim 250$ M^{-1}, and $K_I \sim 5 \times 10^4$ M^{-1} would also explain the values of Na^+ but underestimate the values for Mg^{2+} by about the same amount. Also, for $K_{Cl} < 250$ M^{-1}, decreases in the value of Z for $MgCl_2$ should become detectable. Kowalczykowski et al. (1981) have observed both cation and anion release in the interaction of T4 coded gene 32 protein and polyribonucleotides. There the log–log plots of the effective cooperative binding constant vs. salt concentration are linear, but the slopes vary with cation and anion type.

We cannot distinguish between the simple models outlined above, much less more complicated ones, from the limited data available. We have no information regarding the number of anion binding sites on the repressor, the binding constants of various anions, or the effects of anions on repressor conformation. The only other evidence for anion binding comes from a study using anionic fluorescent probes. York and co-workers (York et al., 1978; Worah et al., 1978) have shown that ANS and bis-ANS bind to *lac* repressor protein and that the fluoresence of the repressor–ANS complex is decreased upon binding of poly(dA-dT). Our results suggest the interpretation that ANS binds to the anion site. Anion effects on proteins are frequently substantial and approximately follow the lyotropic series [see von Hippel & Schleich (1969)], which in order of increasing affinity for proteins is $F^- < SO_4^{2-} < HPO_4^{2-} < CH_3CO_2^- < Cl^- < Br^- < NO_3^- < I^- < CCl_3CO_2^- < ClO_4^- < SCN^-$. In the case of the *lac* repressor–operator interaction, we find the same order, except for the placement of SCN^-, for the monovalent anions F^-, Cl^-, Br^-, NO_3^-, SCN^-, and I^- but no correlation for the carboxylate anions or for the divalent anions. From the few data for pairs of anions, it appears that $CH_3CO_2^-$, Cl^-, and NO_3^- compete for the same site(s). On the other hand, the noncompetitive behavior of Cl^- and I^- would suggest that I^- binds at a different site(s). The amino acid sequence of *lac* repressor protein shows a higher proportion of basic residues in the amino-terminal (residues 1–60) and carboxy-terminal (residues 290–360) regions than in the middle region of the polypeptide (12–14% compared to 6%; Beyreuther, 1978). These basic residues, some of which occur in clusters (residues 33, 35, and 37 and residues 325–327), are potential anion binding sites. However, there is no genetic or biochemical evidence implicating the carboxy-terminal region in DNA binding [see Miller & Reznikoff (1978)]. Because the predominant monovalent anion in *E. coli* is chloride, we do not suppose that the anion effects reported here have a physiological role. Two intracellular polyanions, poly(ADP-ribose) [see Hayaishi & Ueda (1977)] and inorganic polyphosphate [see Kulaev (1979)], are thought to be involved in regulation. Whether such polyanions exert effects on the repressor–operator interaction or on *lac* operon gene expression is unknown. In attempting to interpret our results, we have only considered anion effects on the protein. The effects of anion type on DNA in dilute salt solutions have not been systematically examined. However, Anderson & Bauer (1978) detected no dependence of the duplex rotation angle on anion type. The anion as well as cation effects in concentrated salt solutions arise from differences in hydration of DNA as a function of water activity [see Bloomfield et al. (1974)]. The water activities of our 0.13 M Na^+ buffers are essentially unity (0.9945–0.9957 for the salt; Robinson & Stokes, 1965). It is interesting to note, though, that even as simple a ligand as ethidium binds to DNA with 10-fold lower affinity in nitrate than in chloride (N. Dattagupta, unpublished experiments).

In sum, we have found that the *lac* repressor–operator interaction is extraordinarily sensitive to the ionic environment. In addition to possible biological significance, this has practical implications for in vitro studies of repressor–operator binding. For example, small differences in the binding parameters have been reported for wild-type *lac* repressor proteins isolated from different bacterial strains (Jobe et al., 1972), although sequence analysis suggests that the proteins are identical [see Beyreuther (1978)]. Also, minor differences are observed between repressor preparations from the same strain. This variability could reflect slight differences in the binding buffers or even in the buffers used for purification of the protein and DNA, perhaps due to impurities in the reagents. On the bright side, an enormous range of equilibrium constants becomes accessible by suitable choice of the concentration and type of salt. This should prove useful for physical studies of the *lac* repressor–operator interaction, as well as membrane filter assays of repressors or operators altered by genetical or chemical means.

Comparison of Operator and Nonoperator DNA Binding. The interactions of *lac* repressor with operator and nonoperator DNA are related biological functions of this regulatory protein. As shown in the preceding paper (Barkley, 1981), nonoperator DNA binding plays an important role in the association of repressor to the operator site on a large DNA molecule. Nonoperator DNA binding also serves essential functions in establishing the basal level and in induction of *lac* enzyme synthesis (von Hippel et al., 1974; Lin & Riggs, 1975; Huang et al., 1977). The basis for the repressor's ability to discriminate between operator and nonoperator DNA sequences, which is manifest as about 7 orders of magnitude difference in affinity, is still largely unknown. As mentioned in the introduction, nonoperator DNA binding involves electrostatic interactions between positively charged groups in the DNA-binding site of the repressor and negative phosphate charges on DNA (deHaseth et al., 1977). Operator DNA binding includes a substantial, nonelectrostatic component, amounting to about 9–12 kcal/mol of favorable free energy, in addition to the electrostatic component. The nonelectrostatic binding free energy presumably accounts for the interactions between repressor and specific bases in the operator sequence. Based on their analysis of the data of Riggs et al. (1970b), Record et al. (1977, 1978) have discussed the thermodynamic role of counterion release in the reaction of repressor and operator. Since our results accord with their general conclusions, we will discuss only the added features suggested by this investigation.

In their interpretation of cation effects on the *lac* repressor–operator equilibrium, Record et al. (1977) concluded that there are four more ionic interactions in repressor–DNA complex than in repressor–operator complex. From the salt

dependence of K_{obsd}^{RD}, deHaseth et al. (1977) find $Z_{RD} = 12 \pm 2$ at pH 7.4 and 8.0, and Revzin & von Hippel (1977) find $Z_{RD} \sim 11$ at pH 7.5. From the salt dependence of K_{obsd} at pH 7.4, we find $Z = 10.6 \pm 0.5$; Record et al. (1977) estimate $Z = 8 \pm 1$ from the data of Riggs et al. (1970b) and $Z = 9 \pm 2$ from the data of Lin & Riggs (1975). With this amount of overlap, it seems plausible that the repressor forms about the same number of ionic interactions upon binding operator and nonoperator DNA. Lin & Riggs (1975) have measured operator and nonoperator DNA binding under identical conditions by the membrane filter technique. They find a slightly greater ionic strength dependence for repressor–DNA binding than for repressor–operator binding, which would argue against this interpretation. However, their data at lower ionic strength may not be accurate. The values of K_{obsd} in the presence of 0.003 M Mg^{2+} reported by Lin & Riggs (1975) are almost the same as those in the presence of 0.01 M Mg^{2+} reported by Riggs et al. (1970b). This is not consistent with the competitive effect of Mg^{2+} predicted by Record et al. (1977).

The above estimates of the number of ionic interactions in repressor–operator and repressor–DNA complexes assume no anion release from the protein, since there is no evidence of anion release in the log–log plots of the equilibrium constants vs. cation concentration. However, acetate and chloride exert similar effects on the two equilibria. For example, at [NaX] = 0.13 M, the relative affinities in acetate and in chloride are about 19 for the repressor–operator interaction (Table III) and about 25 for the repressor–DNA interaction (estimated by extrapolation; deHaseth et al., 1977). The effects of other anions on repressor–DNA binding have not been investigated, but the relative effects are probably comparable to those on repressor–operator binding. In the membrane filter experiments, we did not see increased backgrounds in the presence of IPTG for any of the anions. Because repressor–DNA binding is not sensitive to inducer (Revzin & von Hippel, 1977), a decrease in K_{obsd} without a corresponding drop in K_{obsd}^{RD} would have resulted in higher backgrounds on the filters.

Apparently, there are both qualitative and quantitative differences in the pH effects on the repressor–operator and repressor–DNA equilibria. The effects of pH on K_{obsd} are consistent with simple titration of functional groups in the DNA-binding site of the repressor. We find that the number of ionic interactions in repressor–operator complex depends on pH, with $Z = 10.6 \pm 0.5$ at pH 7.4 and $Z = 8.7 \pm 0.6$ at pH 8.0. Moreover, the slopes d log K_{obsd}/(d pH) are small, being in the range −0.9 to −0.3, and K_{obsd} becomes essentially independent of pH at alkaline pH. An alternative explanation for the apparent pH dependence of Z would be that the anion and pH effects are coupled, with more anion release at lower pH. In contrast, the effects of pH on K_{obsd}^{RD} suggest that about two obligatory protonation events accompany binding of repressor and DNA. deHaseth et al. (1977) concluded that the number of ionic interactions in the repressor–DNA complex is independent of pH. At alkaline pH, the slope d log K_{obsd}^{RD}/(d pH) = −2.1 at $[Na^+] = 0.13$ M. Revzin & von Hippel (1977) reported somewhat less pH dependence closer to neutral pH, with a slope of −1.5 at $[Na^+] = 0.15$ M. The binding of *E. coli* RNA polymerase holoenzyme and T7 promoter fragments also appears to require one or two protonation events (Strauss et al., 1980).

Although operator and nonoperator DNA binding respond in like manner to the ionic environment, the subtle differences probably reflect discrete changes in the protein and DNA upon formation of repressor–operator complex.

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Phenylalanyl-tRNA Synthetase of Baker's Yeast. Modulation of Adenosine Triphosphate–Pyrophosphate Exchange by Transfer Ribonucleic Acid†

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ABSTRACT: Native and modified phenylalanine transfer ribonucleic acid ($tRNA^{Phe}$) can modulate phenylalanine-dependent adenosine triphosphate–inorganic [^{32}P]pyrophosphate (ATP–[^{32}P]PP_i) exchange activity via inhibition of adenylate synthesis. Inhibition is visualized if concentrations of L-phenylalanine, ATP, and pyrophosphate are subsaturating. In the proposed mechanism, $tRNA^{Phe}$ is a noncompetitive inhibitor at conditions where only one of the two active sites per molecule of enzyme is occupied by L-phenylalanine, ATP, and pyrophosphate. At saturating concentrations of these reactants, both active sites are occupied and, according to the model, inhibition is eliminated. Occupation by these reactants is assumed to follow homotropic negative cooperativity. The type of effects depends on modification of $tRNA^{Phe}$. Native $tRNA^{Phe}$, $tRNA^{Phe}_{2'\text{-dA}}$, and $tRNA^{Phe}_{oxi\text{-}red}$ are inhibitors, $tRNA^{Phe}$pCpC has no effect, and $tRNA^{Phe}_{ox}$ is an activator. Kinetics of activation by $tRNA^{Phe}_{ox}$ are slow, following the time course of Schiff base formation and subsequent reduction by added cyanoborohydride. Besides showing that a putative enzyme amino group is nonessential for substrate binding and adenylate synthesis, this result may suggest that an enzyme amino group could interact with the 3′-terminal adenyl group of cognate tRNA. In the case of asymmetrical occupation of the enzyme active sites by all of the small reactants ATP, L-phenylalanine, and pyrophosphate, the interaction with the amino group might trigger the observed noncompetitive inhibition of the pyrophosphate exchange by $tRNA^{Phe}$.

Yeast phenylalanine-tRNA synthetase has been demonstrated to bind L-phenylalanine in an anticooperative fashion to its two active sites (Berther et al., 1974; Fasiolo et al., 1977; Fasiolo & Fersht, 1978). The same enzyme from *Escherichia coli* was found to exhibit anticooperativity for both the small substrates, L-phenylalanine and ATP (Pimmer & Holler, 1979). Occupancy of a single site by one of these substrates and presumably also pyrophosphate may be expected to induce asymmetry within the enzyme molecule. An effect of asymmetry can manifest itself as a change in pyrophosphate exchange activity as is reported in the present investigation. If L-phenylalanine, ATP, and pyrophosphate each bind anticooperatively to the enzyme in the absence of the other substrates, it is of interest to see whether the other added substrates can modify this phenomenon. The experiment is to measure the saturation dependence of the pyrophosphate exchange for each substrate varied in the presence of saturating and nonsaturating concentrations of the other substrates. It will be shown that asymmetry is displayed in the presence of cognate tRNA and that asymmetry is eliminated after saturation by any of the substrates L-phenylalanine, ATP, and pyrophosphate.

Materials and Methods

Phenylalanyl-tRNA synthetase from baker's yeast (EC 6.1.1.20) was purified to homogeneity as described previously (Fasiolo & Ebel, 1974). $tRNA^{Phe}$ was purified in our laboratory from total brewer's yeast (Boehringer, Mannheim) by the countercurrent distribution technique. Na[^{32}P]PP_i was a product of the Radiochemical Center (Amersham). [^{14}C]Phenylalanine was from CEA (France). All other reagents were of highest commercially available purity.

ATP–PP_i Exchange Reaction. All the experiments described have been performed at 25 °C in a standard buffer containing 144 mM Tris-HCl, pH 7.8, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 0.1 mM phenylmethanesulfonyl fluoride. Final concentrations for the ATP–PP_i exchange assay are specified in the figure legends. The initial rates of exchange

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