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Nonspecific Interaction of *lac* Repressor with DNA: An Association Reaction Driven by Counterion Release†

Pieter L. deHaseth, Timothy M. Lohman, and M. Thomas Record, Jr.*

ABSTRACT: We have investigated the nonspecific interaction of *lac* repressor protein with DNA by a quantitative application of DNA-cellulose chromatography (deHaseth, P. L., et al. (1977), *Biochemistry 16* (third of five papers in a series in this issue)). The observed association constant for the interaction, K^{RD}_{obsd}, is a sensitive function of ion concentrations and pH. Application of binding theory to interpret these effects gives the results that 11 ± 2 monovalent ions are released in the interaction and two groups on repressor must be protonated for repressor to bind to DNA. We argue that much of the ion release results from the displacement of cations from the DNA, and estimate on this basis that 12 ± 2 phosphates are involved in ionic interactions with the protein. Ion release drives the protonation reaction and the overall repressor-DNA interaction. The major role of low molecular weight ions in the repressor-DNA interaction suggests that ion concentration changes must be considered in discussing mechanisms of control of gene expression.

Many of the association reactions of proteins are driven by the release of structured water and show the thermodynamic behavior ascribed to the hydrophobic effect (Lauffer, 1974; Tanford, 1973). A different principle is operative in the interactions of highly charged biopolymers. Analysis of the interaction of various charged ligands (Mg^{2+}, oligolysines, RNase) with nucleic acids (Record et al., 1976a) demonstrated that these association reactions are driven by the entropic effect of release of monovalent cations from the nucleic acid. The entropic effect of ion release plays a major role in the aggregation of myosin (Josephs and Harrington, 1968) and of *Escherichia coli* RNA polymerase (Wensley and Record, in preparation), and is a factor in the allosteric transition of hemoglobin (Shulman et al., 1975) and the helix coil transitions of nucleic acids (Record et al., 1976b).

Here we give an experimental study and theoretical analysis of the thermodynamics of the nonspecific interaction of *lac* repressor protein with DNA.[1] The analysis is general and should be applicable to any charged ligand-nucleic acid interaction. We find that the fundamental driving force for the association reaction is the release of a large number of counterions from the nucleic acid and perhaps also from the protein. Counterion release, we will show, drives a protonation reaction of repressor which is necessary for formation of the nonspecific complex. The implications of this study for the repressor-operator interaction are considered in the following paper (Record et al., 1977).

The participation of a large number of ions in the repressor-DNA interaction means that the extent of binding of repressor to nonspecific DNA sites is extremely sensitive to small changes in ionic conditions. It seems plausible to suggest that ion concentration changes in vivo may play some role in modulating the control of gene expression.

Theoretical Section

Consider the interaction of repressor with nonoperator DNA to form the nonspecific complex RD. The observed reaction is

$$R + D \overset{K^{RD}_{obsd}}{\rightleftharpoons} RD \qquad (1)$$

where

$$K^{RD}_{obsd} = \frac{[RD]}{[R][D]} \qquad (2)$$

and where [R] and [D] are *total* molar concentrations of free repressor and free DNA nucleotides, irrespective of titration state or degree of ligation of low molecular weight ions.

The association constant K^{RD}_{obsd} is found to be a function of temperature, pH, and monovalent and divalent ion concentrations in the reaction mixture (see below; also Revzin and von Hippel, 1977). These dependences indicate that small ions and titrated forms of R and/or D participate in the molecular association reaction. Analysis of the ion and pH dependences of K^{RD}_{obsd} using binding theory (Wyman, 1964; Schellman, 1975) allows one to obtain the numbers of participating ions, and provides information about the molecular details of the binding reaction. For example, Riggs (1971) has analyzed the pH dependence of the binding of 2,3-diphosphoglycerate to hemoglobin to determine the pK and number of titrating groups in this reaction and has shown that the apparent enthalpy of the observed association reaction results primarily from the large enthalpies of titration of these groups. An analogous approach is common in steady-state enzyme kinetics, where the pH dependence of the reaction velocity pro-

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[1] Preliminary accounts of this work were presented to the Biophysical Society (Seattle, 1976; New Orleans, 1977) and at the Cold Spring Harbor meeting on Molecular Aspects of *lac* Operon Control (1976). Some of our present conclusions supercede the results of those preliminary theoretical analyses.

vides information about the pK and number of groups that titrate in the formation of the enzyme–substrate complex (cf. Segel, 1975).

Recently we developed the corresponding treatment of monovalent ion effects on the interactions of small charged ligands (e.g. Mg^{2+}, oligolysines) with nucleic acids (Record et al., 1976a). One can obtain the number of ionic interactions between ligand and nucleic acid by this approach; for a series of oligolysines studied by Latt and Sober (1967), we found that the number of ionic interactions (ion pairs) was equal to the number of charged lysine residues in the oligomer. Central to the theory is the idea of a thermodynamically bound counterion, the consequence of the high axial charge density on the nucleic acid. Counterions participate in condensation and screening interactions with the polyelectrolyte to an extent determined by the axial charge density alone (Manning, 1969). These interactions are thermodynamically equivalent to the binding of the fraction ψ of a monovalent cation (M^+) to each phosphate (Record et al., 1976a). Neutralization of a phosphate by ion-pair formation to a ligand releases ψ M^+ ions. If there are no complications from anion binding by the ligand or titration or hydration effects, then the theory predicts that

$$-\left(\frac{\partial \log K_{obsd}}{\partial \log [M^+]}\right) = m'\psi \tag{3}$$

where m' is the number of ion pairs between ligand and nucleic acid. For double helical DNA, ψ is known to be 0.88 (Record et al., 1976a,b); consequently m' may be determined from the dependence of log K_{obsd} on log $[M^+]$.

Here we extend the above analysis to treat ion and pH effects on the repressor–DNA interaction, and on protein–nucleic acid interactions generally. We include the possibility of anion binding by the DNA binding site of the protein, the titration of groups in that site, and the effects of added Mg^{2+} (instead of Na^+) on the binding equilibrium. The situation when Na^+ and Mg^{2+} are jointly present in the binding buffer is considered in the next paper (Record et al., 1977).

In the presence of excess univalent salt (M^+, X^-), it is convenient to define a thermodynamic association constant K^{RD,M_T} for the molecular reaction

$$R_{r,0} + D_0 \overset{K^{RD,M_T}}{\rightleftharpoons} RD + m'\psi M^+ \tag{4}$$

where

$$K^{RD,M_T} \equiv \frac{[RD][M^+]^{m'\psi}}{[R_{r,0}][D_0]} \tag{5}$$

Here $[R_{r,0}]$ is the molar concentration of repressor species with r protons and zero anions bound to regions involved in the DNA binding reaction. $[D_0]$ is the molar concentration of DNA nucleotides with ψ M^+ ions associated (in the thermodynamic sense, as discussed above) but with no other associated ligands. Equation 4 is an appropriate reference equation because it has a simple form and an invariant stoichiometry. The effect of anion binding equilibria is to reduce $[R_{r,0}]$; an increase in pH will also reduce $[R_{r,0}]$. The presence of a DNA binding ligand like Mg^{2+} in addition to the monovalent ions M^+ (see Record et al., 1977) will reduce $[D_0]$. (We want to emphasize that this is a thermodynamic description of the *lac* repressor–DNA interaction, without any implication for the actual mechanism of the binding reaction.) All of the above effects can be expressed in secondary equilibria. For eq 4 to be useful, however, we require that only the r-protonated form of repressor binds to DNA, and that no other cations (M^+, Mg^{2+}) or anions are required for the interactions between R and D in the RD complex.

When only monovalent ions are present, $[D] = [D_0]$, and combining eq 2 and 5 we get:

$$K^{RD,M_T} = K^{RD}{}_{obsd}\frac{[R]}{[R_{r,0}]}[M^+]^{m'\psi} \tag{6}$$

The ratio $[R]/[R_{r,0}]$ can be evaluated analytically if certain simplifying assumptions are made.

$$\frac{[R]}{[R_{r,0}]} = \frac{[R]}{[R_r]}\frac{[R_r]}{[R_{r,0}]} \tag{7}$$

where $[R_r]$ is the concentration of repressor with r protons and any number of anions bound. (Note that the only binding sites for protons and anions of concern to us are those which are also involved in the binding of DNA. Consequently the specification R_r or $R_{r,0}$ does not uniquely define the total amount of anion or proton binding in these forms.) Designate the intrinsic binding constants for each of the r protons as K_H. Then, if the r sites are independent (Schellman, 1975),

$$\frac{[R]}{[R_r]} = \left[\frac{1 + K_H[H^+]}{K_H[H^+]}\right]^r \tag{8}$$

If the species $R_{r,0}$ has a independent and identical binding sites for anions, with an intrinsic binding constant K_X, then (Schellman, 1975)

$$\frac{[R_r]}{[R_{r,0}]} = (1 + K_X[X^-])^a \tag{9}$$

Combining eq 6–9 and taking logarithms, we obtain the following general expression for the pH and ion concentration dependences of $K^{RD}{}_{obsd}$:

$$\log K^{RD}{}_{obsd} = \log K^{RD,M_T} - r \log\left[\frac{1 + K_H[H^+]}{K_H[H^+]}\right] - a \log(1 + K_X[X^-]) - m'\psi \log [M^+] \tag{10}$$

A number of special cases of eq 10 are of interest.

(a) In the absence of titration effects and anion binding, eq 10 reduces to the equation previously used by Record et al. (1976a) to analyze the interactions of model ligands (Mg^{2+}, oligolysines) with nucleic acids:

$$\log K_{obsd} = \log K_T - m'\psi \log [M^+] \tag{11}$$

In the particular case of Mg^{2+} as a ligand (as opposed to its role as a competitor; Record et al., 1977), then eq 11 is applicable with $m' = 2$ and a thermodynamic binding constant $K^{Mg}{}_T$ which depends on the nature of the nucleic acid lattice (Record et al., 1976a).

(b) At constant pH, differentiation of eq 10 with respect to log $[M^+]$ gives

$$-\left(\frac{\partial \log K^{RD}{}_{obsd}}{\partial \log [M^+]}\right) = m'\psi + \frac{aK_X[X^-]}{1 + K_X[X^-]} \tag{12}$$

since $[M^+] \simeq [X^-]$. (MX is added in excess over all other ionic species.) Here $m'\psi$ is the number of cations released from the DNA on binding repressor, and $aK_X[X^-]/(1 + K_X[X^-])$ is the number of anions released by repressor on binding to DNA. This latter term was designated k by Record et al. (1976a). Note that the presence of anion binding will introduce curvature into a plot of log $K^{RD}{}_{obsd}$ vs. log $[M^+]$; under such conditions extrapolation of binding data must be carried out using the functional form of eq 10. Note also that the presence of anion binding introduces two parameters (a and K_X) into the binding equation. Without independent information about these parameters, an accurate value of m' cannot be obtained from the $[M^+]$ dependence of log $K^{RD}{}_{obsd}$, unless data of high

precision are available over a wide range of $[M^+]$.

(c) At a constant concentration of MX, the pH dependence of K^{RD}_{obsd} can provide molecular information about the number (r) and intrinsic association constant (K_H) of groups on the repressor that must take up a proton above their normal pK in order for the repressor to bind to DNA. By differentiation of eq 12 with respect to pH at constant ion concentration and temperature, we obtain

$$-\left(\frac{\partial \log K^{RD}_{obsd}}{\partial \text{ pH}}\right) = \frac{r}{1 + K_H[H^+]} \tag{13}$$

At sufficiently alkaline pH, $\log K^{RD}_{obsd}$ is predicted to be a linear function of pH with a slope of $-r$, since $K_H[H^+] \ll 1$. At the point where $K_H[H^+] = 1$, which is the pK of the titrating groups, the magnitude of the slope is one-half its alkaline value. At sufficiently low pH, K^{RD}_{obsd} becomes independent of pH because all participating groups are fully protonated. If we define $\Delta \log K^{RD}_{obsd} \equiv \log K^{RD}_{obsd}$ (low pH limit) $- \log K^{RD}_{obsd}$, then from eq 10 at constant ion concentrations and temperature

$$\Delta \log K^{RD}_{obsd} = r \log \left[\frac{1 + K_H[H^+]}{K_H[H^+]}\right] \tag{14}$$

Equation 14 is useful in predicting the low pH limit of K_{obsd} of a pH dependent binding equilibrium, and gives the reduction in K^{RD}_{obsd} to be expected from the requirement for protonation of groups at a pH above the normal group pK.

The coupling of the protonation reaction to the R-D binding reaction also results in an observed reaction enthalpy that is a function of r and pH:

$$\Delta H^\circ_{obsd} = \Delta H^\circ_T + \frac{r\Delta H^\circ_{pro}}{1 + K_H[H^+]} \tag{15}$$

where

$$\Delta H^\circ_{obsd} = -R \frac{\partial \ln K^{RD}_{obsd}}{\partial\, 1/T}$$

$$\Delta H^\circ_T = -R \frac{\partial \ln K^{RD}_T}{\partial\, 1/T}$$

and

$$\Delta H^\circ_{pro} = -R \frac{\partial \ln K_H}{\partial\, 1/T}$$

Thus in the low pH limit defined above, $\Delta H^\circ_{obsd} = \Delta H^\circ_T$; in the high pH region where $K_H[H^+] \ll 1$, $\Delta H^\circ_{obsd} = \Delta H^\circ_T + r\Delta H^\circ_{pro}$. Studies of the pH and temperature dependence of K^{RD}_{obsd} can therefore be used to identify the number and chemical identity of the protonating groups on repressor.

To help remove ambiguities due to anion binding from the determination of m', it is convenient to carry out protein-nucleic acid binding experiments in the presence of MgX_2 only. This will shift the range of X^- concentrations associated with experimentally accessible values of K^{RD}_{obsd} to substantially lower concentrations, and thereby reduce the importance of anion binding. To interpret experiments in MgX_2, replace molecular reaction 4 by

$$R_{r,0} + D \overset{K^{RD,Mg_T}}{\rightleftharpoons} RD + m'\phi Mg^{2+} \tag{16}$$

where

$$K^{RD,Mg_T} = \frac{[RD][Mg^{2+}]^{m'\phi}}{[R_{r,0}][D]} \tag{17}$$

Here we use the total concentration of nucleotides [D] in eq 17 because the fraction ϕ of a Mg^{2+} ion is associated in the thermodynamic sense with *each* nucleotide, in the absence of M^+, or other ligands. Application of polyelectrolyte theory (Manning, 1969), in a manner parallel to that used by Record et al. (1976a) to evaluate ψ, leads to a value of ϕ of 0.47 for double helical DNA. K^{RD,Mg_T} is the thermodynamic equilibrium constant for reaction 16; $m'\phi$ is the generalized stoichiometric coefficient of Mg^{2+}. By analogy with eq 10, in the absence of M^+ ions

$$\log K^{RD}_{obsd} = \log K^{RD,Mg_T} - r \log \left[\frac{1 + K_H[H^+]}{K_H[H^+]}\right] - a \log(1 + K_X[X^-]) - m'\phi \log [Mg^{2+}] \tag{18}$$

At constant temperature and pH

$$-\left(\frac{\partial \log K^{RD}_{obsd}}{\partial \log [Mg^{2+}]}\right) = m'\phi + \frac{aK_X[X^-]}{1 + K_X[X^-]} \tag{19}$$

since $[Mg^{2+}] = (1/2)[X^-]$. In the limit of no anion binding, comparison of eq 12 and 19 yields the relationship

$$\left|\left(\frac{\partial \log K^{RD}_{obsd}}{\partial \log [Mg^{2+}]}\right)\right| = 0.53 \left|\left(\frac{\partial \log K^{RD}_{obsd}}{\partial \log [M^+]}\right)\right| \tag{20}$$

The numerical factor 0.53 is the ratio ϕ/ψ for helical DNA.

A consistency relationship can be obtained between the thermodynamic constants K^{RD,M_T}, K^{RD,Mg_T}, and K^{Mg_T} using the formalism developed by Record et al. (1977) to describe the effects of M^+ and Mg^{2+} when these are jointly present in the binding buffer, and taking the limit of low $[M^+]$ and high $[Mg^{2+}]$.

$$\log K^{RD,M_T} = \log K^{RD,Mg_T} + \frac{m'}{2} \log K^{Mg_T} \tag{21}$$

(To derive eq 21, ϕ is approximated by the value ½.) Equation 21 should provide a check on the internal consistency of the various K_T values for protein and Mg^{2+} binding to DNA.

At this point it is appropriate to emphasize that we have chosen the simplest cases (independent, identical sites for binding of anions to repressor, independent and identical sites for binding of protons to repressor, no differential hydration effects, no specific involvement of ions in the RD complex) to evaluate using the binding theory. At present this is necessary because we have no independent knowledge of the parameters m', a, r, and the various thermodynamic binding constants. When such information becomes available, the binding theory can be applied in a less approximate form. The theory in its present form also neglects several effects of ligand binding on the polyelectrolyte properties of the DNA, including (a) possible site binding of low molecular weight ions to fixed charges which are covered but not neutralized in the RD complex, (b) probable reduction in the ion association requirements of DNA phosphate across the helix from the region of repressor binding, (c) possible interactions between the repressor surface charge distribution and the DNA, exclusive of ion pairing, and (d) the expected slight decrease in ψ with increased ligand binding density on the DNA. The contribution of effects a and b can be estimated using the approach of Record et al. (1976a), if the overlap site size n (McGhee and von Hippel, 1974) and number of ion pairs m' are known. In the case of repressor, for which $n \simeq 24$ nucleotides (Butler et al., 1977; Maurizot et al., 1974) and the maximum m' is estimated to be 12 ± 2 (see below) the maximum error in m' from neglect of effects a and b is 10–15%. This uncertainty is comparable to the experimental uncertainty in obtaining m' from the slope of the log K^{RD}_{obsd} vs. log $[M^+]$ plot and the effects are therefore neglected.

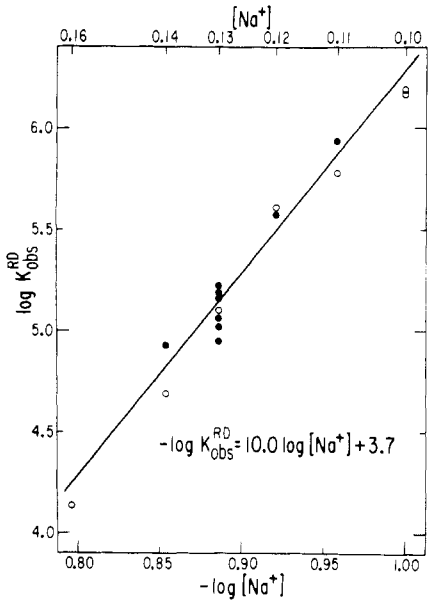

FIGURE 1: The dependence of the observed binding constant of the *lac* repressor-DNA interaction on the concentration of added NaCl; log-log plot. Elution at 4 °C with buffer T (pH 8.0). (●) Wisconsin prep; 87 μg loaded per run. (○) Harvard prep; 100 μg loaded per run. See deHaseth et al. (1977) for a description of the two preparations.

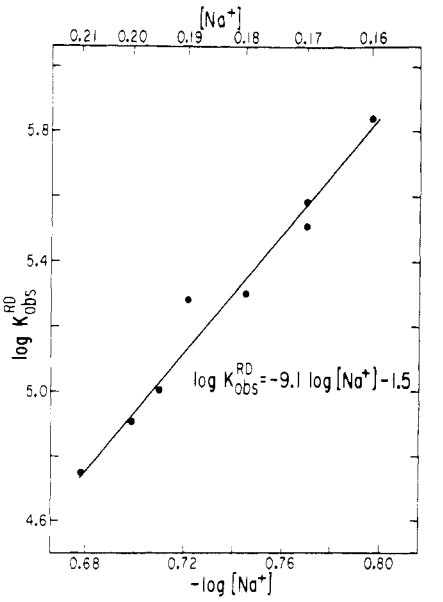

FIGURE 2: The dependence of the observed binding constant of the *lac* repressor-DNA interaction on the concentration of added sodium acetate; log-log plot. Elution at 4 °C with buffer T (pH 8.0); 87 μg of repressor loaded per run.

Materials and Methods

The procedures have been described in the preceding paper (deHaseth et al., 1977). The use of DNA-cellulose columns to determine binding constants of protein-DNA interactions is described in detail therein. Buffer P and buffer T are as outlined by deHaseth et al. (1977). Two additional buffers have been used here, differing from the above buffers only in the concentration and nature of the buffering agent. Thus 0.001 M Tris buffer is 0.001 M Tris (pH 8.0 at 4 °C), 7×10^{-3} M β-mercaptoethanol, 10^{-4} M EDTA, 5% glycerol, and 0.005 M Bicine[2] buffer is 0.005 M Bicine (Sigma) (pH 8.0 at 4 °C), 7×10^{-3} M β-mercaptoethanol, 10^{-4} M EDTA, 5% glycerol.

The two preparations of *lac* repressor used in this study have been described in the preceding paper (deHaseth et al., 1977). In our initial experiments we used the Harvard preparation; in this paper only Figure 1 contains data obtained using this preparation. Plots of the logarithm of the percentage of protein initially loaded which remains on the column after elution of a fraction vs. fraction number showed deviations from linearity for the Harvard preparation in the mid-region of the elution (deHaseth et al., 1977). Binding constants were then obtained from the slopes of tangent lines to the semilog plots, drawn through the point where 60% of the protein had not eluted. The choice of the "60%" point does not affect the calculated dependence of the observed binding constant on variables like the salt concentration; it does have an influence on its absolute magnitude, however. The curvature is such that the binding constant calculated at the "30%" point is generally twice as large as the one calculated at the "80%" point. The "60%" point was chosen so that the binding constants calculated this way agreed in absolute value with those obtained with the Wisconsin preparation (with linear semilog plots).

Results

(1) *Formation of the RD Complex in the Presence of MX Releases 11 ± 2 Salt Ions.* The dependence of K^{RD}_{obsd} on monovalent electrolyte concentration has been determined in various buffers, using Na^+ as the cation and either Cl^- or CH_3COO^- as the anion. In every case, log K^{RD}_{obsd} is a linear function of log [Na^+], as predicted by eq 11 or 12. Strict interpretation of eq 12 would indicate curvature should be observed in these plots if the release of anions from the repressor is a factor in the reaction. However, the range of salt concentrations studied is narrow and the data are not sufficiently accurate to determine whether or not some curvature is present.

Our data and that of Revzin and von Hippel (1977) for the effect of NaCl concentration on K^{RD}_{obsd} at 20 °C in phosphate buffer were shown as Figure 7 of the preceding paper (deHaseth et al., 1977). The slopes $\partial \log K_{obsd}/\partial \log [Na^+]$ were −12.0 and −10.1, respectively, implying that an average of 10–12 ions are released in the RD interaction in the salt range and buffer studied. Figures 1 and 2 show the results of series of experiments in Tris buffer (0.01 M, pH 8, 4 °C) using NaCl (some of which were shown as Figures 2 and 3 of deHaseth et al. (1977)) and CH_3CO_2Na as the monovalent salt. The least squares slopes are −10.0 and −9.1, respectively. Data in 0.005 M Bicine buffer, pH 8, give a slope of −12.1 for NaCl as the electrolyte (Record et al., 1977). Measurements on the interaction between *lac* repressor and T7 DNA using a differential boundary sedimentation velocity technique (Lohman and Record, unpublished experiments) give a slope of −11.3 in 0.01 M Tris, pH 7.5, 20 °C; [NaCl] was variable. Consequently all these data are consistent with the release of 11 ± 2 ions in the formation of the RD complex.

In principle, the intercepts of these plots at log [Na^+] = 0 (the 1 M standard state) would provide estimates of the nonelectrostatic contributions to the free energy of association. However, if anion release or titration effects are involved in the interaction, the extrapolation to the standard state is not straightforward and requires knowledge of the various terms in eq 10. There are pH, anion, and even buffer effects on K^{RD}_{obsd}, as will be discussed below, and consequently the intercepts are not simply interpreted.

[2] Abbreviations used: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; IPTG, isopropyl β-D-thiogalactoside; pro, protonation.

If the contribution of anion release to the total observed ion release in the RD interaction is small, then m', the number of phosphate groups involved in ionic interactions with repressor, can be estimated to be 12 ± 2. This is a maximum estimate, obtained from eq 12. If there is detectable anion release, then m' is less than this value.

An anion effect on K^{RD}_{obsd} is apparent in a comparison of Figures 1 and 2. All conditions in these two sets of experiments were identical, except that in one case the anion was Cl^-, in the other CH_3COO^-. Values of K^{RD}_{obsd} are approximately 40 times larger in acetate than in chloride at the same total ion concentration. The difference is far outside the error limit in K^{RD}_{obsd}, estimated to be a factor of two or less (deHaseth et al., 1977). In the case of the interaction of pentalysine with T7 DNA (Lohman and Record, unpublished experiments), the observed binding constant is not affected by the replacement of Cl^- with CH_3COO^-. The 40-fold difference in K^{RD}_{obsd} can be explained if Cl^- ions bind to the DNA binding region of repressor much more strongly than CH_3COO^- ions do. However, the fact that the slopes of the lines in Figures 1 and 2 differ by only 10% (which is within the standard error of either least-squares line) indicates that there are no major differences in the amount of anion release (if any) in the two systems. As a possible but not unique interpretation of the data, the presence of one Cl^- binding site with an association constant of 250 M^{-1}, to which acetate ion does not bind, would explain the effect. From eq 10, the presence of this site would reduce K^{RD}_{obsd} by a factor of (1 + 250 [Cl^-]), or approximately a factor of 40 at 0.16 M NaCl. Moreover the release of Cl^- from this site would increase $-\partial \log K_{obsd}/\partial \log [Na^+]$ by approximately one, since this derivative counts the number of ions released in the RD interaction. This also is consistent with the data. If only one Cl^- ion is released in NaCl, curvature would not be detected in the log-log plot of Figure 1.

There is an alternative explanation of the acetate effect, which is equally plausible. Acetate may bind to the protein at sites not directly involved in DNA binding and induce a conformational change in repressor which enhances the affinity of the protein for DNA. A parallel situation to this would be the binding of antiinducers, which increase the affinity of repressor for operator (Bourgeois and Pfahl, 1976). To our knowledge, neither the effect of acetate on the repressor-operator interaction nor the effect of antiinducers on the repressor-DNA interaction has been studied. However, inducers of the *lac* operon, like isopropyl thiogalactoside (IPTG), do not affect the nonspecific repressor-DNA interaction (Revzin and von Hippel, 1977); moreover both free and DNA-bound (RD) repressor show the same affinity for IPTG (Friedman et al., 1977).

Comparison of the data in 0.01 M Tris (Figures 1 and 2) with that obtained in phosphate (Figure 7 of deHaseth et al., 1977) or in Bicine suggests that the presence of Tris reduces the total number of Na^+ and Cl^- ions released slightly, as compared with the situation in phosphate or Bicine. This would occur if some Tris cations condense on the DNA in place of Na^+. Since Na^+ was present in at least 20-fold excess over the Tris cation, interference by the bulky Tris ion was not expected to be a problem. Tris was originally used as the buffer to facilitate comparison with the repressor-operator binding buffer of Riggs et al. (1970a,b). We have subsequently avoided cationic buffers.

(2) *Formation of the RD Complex in $MgCl_2$ Releases 6 ± 1 Ions.* The dependence of log K^{RD}_{obsd} on log [Mg^{2+}] in either 0.001 M Tris buffer or 0.005 M Bicine buffer, pH 8, is linear, within experimental error. As indicated in Figure 3, the two sets of data are superimposable, and a least-squares line has

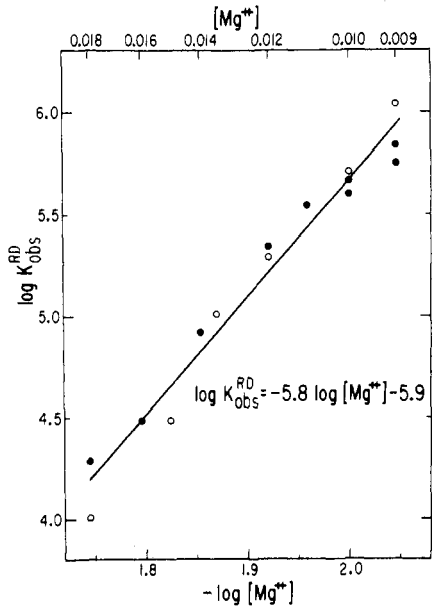

FIGURE 3: The dependence of the observed binding constant of the *lac* repressor-DNA interaction on the concentration of added $MgCl_2$; log-log plots. Elution at 4 °C; 150 μg of repressor loaded per run. (●) Elution with 0.005 M Bicine buffer (pH 8.0). (○) Elution with 0.001 M Tris buffer (pH 8.0).

been fit to the entire data set. Linear behavior of log K_{obsd} vs. log [Mg^{2+}] is predicted by eq 19, in the absence of monovalent cations and provided that the anion binding effect is not large. Note that the concentrations used in these experiments (0.009-0.02 M) are approximately an order of magnitude less than the range of Na^+ concentrations used in the experiments of Figures 1 and 2. This can be understood by reference to eq 21, 12, and 19. For the interaction of Mg^{2+} with poly(A)·poly(U), log $K^{Mg}_T \simeq 1$ from the analysis of Record et al. (1976a) of the data of Krakauer (1974). Our results on the Mg^{2+}-DNA interaction (Record et al., 1977) give a range of smaller but still positive values of log K^{Mg}_T. Consequently, from eq 21, log $K^{RD,Mg}_T$ < log $K^{RD,M}_T$. Moreover from eq 12, 19, and 20, the slope of a log-log plot of K^{RD}_{obsd} vs. ion concentration must be smaller in magnitude in $MgCl_2$ than in NaCl. Therefore the concentration of $MgCl_2$ at which K^{RD}_{obsd} has a particular value (say 10^5 M^{-1}) will be much lower than the NaCl concentration required to give that same value of K^{RD}_{obsd}. The qualitative physical picture is that the formation of the RD complex requires counterion displacement, and is driven by the entropic effect of counterion release. Mg^{2+} ions are associated more tightly with the DNA than Na^+ ions, so more free energy is expended in displacement of Mg^{2+}; in addition only approximately half the number of ions are released, so the entropic contribution to the free energy of interaction is only half as great at any given salt concentration. Other factors (such as the effects of Na^+ or Mg^{2+} ions on the conformation of the protein) may also contribute.

From the slope of the least-squares line in Figure 3, we conclude that 6 ± 1 ions are released in the formation of the RD complex in $MgCl_2$. Recall that, in NaCl, 11 ± 2 ions were released. Although the uncertainty limits are too large to permit us to draw a definite conclusion, these results are consistent with the situation (cf. eq 20) in which there is no anion binding. In this case, the ratio of ion release terms should be 0.53; the experimental value is 0.55 ± 0.19. We can rule out the possibility of a large number of anion sites with low binding affinities. We cannot rule out a small number of anion sites with either low or high affinity. At the present, the simplest

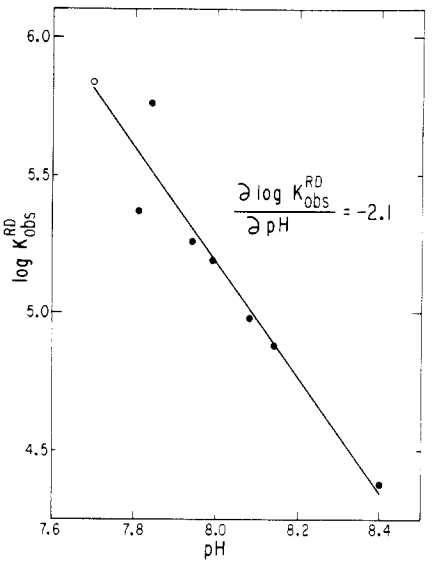

FIGURE 4: The dependence of the observed binding constant of the *lac* repressor–DNA interaction on pH. Elution at 4 °C in buffers containing 0.13 M NaCl. Repressor, 87 μg, loaded per run. (●) Buffer T, pH variable. (○) Buffer P, pH 7.70.

interpretation of our data is that anion release is not a major factor in the thermodynamics of the reaction. This assumption eliminates two parameters (a, K_X) from eq 10 and simplifies the subsequent analysis, but should not be regarded as more than a reasonable working hypothesis.

(3) *The RD Interaction in the pH Range 7.7–8.4 Requires That Two or More Groups on Repressor Protonate at a pH above Their Normal pK.* The pH dependence of K^{RD}_{obsd} in 0.13 M NaCl is shown in Figure 4. Over the pH range 7.7–8.4, log K^{RD}_{obsd} is a linear function of pH, with a slope of -2.1 ± 0.2. This behavior is consistent with that predicted by eq 13 for two independent and identical sites of protonation in the pH range sufficiently above the normal group pK so that $K_H[H^+] \ll 1$. Then $-\partial \log K^{RD}_{obsd}/\partial pH = r$, and $r = 2$.

In light of the finding of Wu et al. (1976) that repressor undergoes a conformational transition between two states accompanied by the cooperative uptake of two protons (or the less cooperative uptake of 3–4 protons), we have considered the effect of assuming cooperative uptake of r protons. In this case eq 8 is replaced by

$$\frac{[R]}{[R_r]} = \frac{1 + K_H{}^r[H^+]^r}{K_H{}^r[H^+]^r} \qquad (8')$$

and eq 13 becomes

$$-\left(\frac{\partial \log K_{obsd}}{\partial pH}\right) = \frac{r}{1 + K_H{}^r[H^+]^r} \qquad (13')$$

Consequently the behavior of Figure 4 is equally consistent with that of a cooperative uptake of two protons in the region $K_H[H^+] \ll 1$ (i.e., pH $\gg$ pK).

Qualitatively, the reason K^{RD}_{obsd} increases so dramatically with decreasing pH at constant salt concentration is that less of an expenditure of free energy is required to protonate the two groups as the pH approaches the group pK. If protonation occurred solely to permit the formation of two additional ionic interactions with the DNA, with the accompanying release of 2ψ Na^+ ions, then at sufficiently alkaline pH the protonation reaction should not occur, because a point will be reached where the free energy expenditure for protonation is not recovered in the subsequent ion pairing and counterion-release steps. This is not observed; log K^{RD}_{obsd} decreases linearly with pH over the range studied, although it is possible that deviations from linear behavior would occur at more alkaline pH. Alternatively, the uptake of the two protons may be coupled to a conformational change in repressor that is required for the nonspecific binding interaction with DNA. This second alternative is made more attractive by its agreement with the model of Wu et al. (1976). It is possible that the form of repressor which Wu et al. (1976) implicate in the binding of protons ($K_H = 2 \times 10^7$ M^{-1}) and the inducer isopropyl thiogalactoside (IPTG) is the form which participates in nonspecific binding. At pH 7.7, the lowest pH in our series (Figure 4), $K_H{}^2[H^+]^2 = 0.16$, and a linear plot of log K^{RD}_{obsd} vs. pH would be expected above this pH from eq 13′, in agreement with Figure 4. If this interpretation is correct, however, then IPTG should increase the nonspecific binding of repressor at a given pH, since IPTG shifts the conformational equilibrium to favor the protonated form of Wu et al. (1976). As noted previously, IPTG has been observed to have no effect on nonspecific binding (Revzin and von Hippel, 1977). Further experiments over a wider pH range are in progress to understand the molecular origin of the pH effect.

Table I: Temperature Dependence of the *lac* Repressor–DNA Binding Constant.[a]

Temp (°C)	Log K^{RD}_{obsd}
4	5.96
4	5.80
21	5.65
22	5.59
36	4.89
38	4.95

[a] Experiments in buffer $P_{0.13}$ (see deHaseth et al., 1977), pH 7.7.

(4) *The RD Interaction at Sufficiently Alkaline pH Is Accompanied by a Negative Enthalpy Change Resulting from the Enthalpy of Protonation of Two Groups on the lac Repressor.* The temperature dependence of K^{RD}_{obsd} at fixed [NaCl] and pH (0.13 M NaCl, pH 7.7) is given in Table I. The van't Hoff plot of these data appears nonlinear on the basis of six points, although more experiments are needed to accurately delineate the shape of the curve. If we ignore the curvature and fit the data of Table I to a least-squares line, we obtain $\Delta H°_{obsd} = -11$ kcal/mol from the slope. The uncertainty in this estimate is approximately ±50%, with a less negative $\Delta H°_{obsd}$ appropriate to the lower temperature data points. For comparison, Revzin and von Hippel (1977) obtain a reaction enthalpy of −6 kcal at pH 7.5 over the range 10–30 °C.

These negative values for $\Delta H°_{obsd}$ seem inconsistent with our statement that the release of ions from the DNA and perhaps also from repressor provides the major driving force for the association reaction. This apparent inconsistency is resolved when one recalls that at the pH of the temperature series the uptake of approximately two protons is coupled to the binding reaction. Consequently, from eq 15, in the alkaline pH range, $\Delta H°_{obsd} \simeq \Delta H°_T + 2H°_{pro}$. (If the Wu et al. (1976) model is applicable, then an additional term in this equation is necessary to account for the enthalpy of the conformational transition of repressor.) The two titrating groups are probably histidines or α-NH_2 groups. The participation of the N terminal α-NH_2 groups of two subunits of the repressor tetramer is particularly attractive, in view of genetic evidence (Müller-Hill et al., 1975) implicating this region in operator binding, and by close analogy with the behavior of the binding site for 2,3-diphosphoglycerate (DPG) on hemoglobin (Riggs, 1971). According to Shiao and Sturtevant (1976), the enthalpies of protonation of histidine and α-NH_2 groups are

−6.3 and −10.0 kcal/mol, respectively. Therefore $\Delta H°_{pro}$ may well dominate $\Delta H°_{obsd}$; $\Delta H°_{T}$, which refers to the enthalpy of the reaction of titrated repressor with DNA, may be small or even positive (see below).

Discussion

(1) *The Thermodynamic Parameters of the RD Interaction in NaCl.* The experiments discussed above have demonstrated that the observed association constant K^{RD}_{obsd} is very sensitive to ionic concentrations, pH, and temperature. Apparently any value of K^{RD}_{obsd}, and therefore of the observed free energy change $\Delta G°_{obsd}$, can be obtained by a suitable choice of ionic conditions. It is therefore important to attempt to obtain K^{RD}_{T}, the thermodynamic association constant, which is independent of ion concentrations and pH. Equation 10, evaluated in the absence of Mg^{2+}, provides the prescription for doing this. However, eq 10 contains one totally unknown parameter (K_H, the protonation constant) and several incompletely known quantities (m', a, K_X). Moreover, if an allosteric transition is a part of the binding mechanism, then it must be incorporated into the formalism of eq 7-11. Nevertheless we give here a range of estimates of $\log K^{RD}_{T}$, based on reasonable estimates of the parameters involved. We choose $K_H = 1.6 \times 10^7\ M^{-1}$, corresponding to a p$K$ of 7.2 at 4 °C, which agrees with the temperature-corrected best-fit pK of the α-NH_2 group used by Lohman and Record (unpublished) in the application of eq 10 to the interaction of pentalysine with T7 DNA. If $a = 0$, $r = 2$, and $m'\psi = 11 \pm 2$, then, since $\log K^{RD}_{obsd} \simeq 5.1$ at pH 8, 0.13 M NaCl, we obtain $\log K^{RD,Na}_{T} \simeq -2.9 \pm 1.7$. If $a = 1$, $K_X = 250\ M^{-1}$, $r = 2$, $K_H = 1.6 \times 10^7\ M^{-1}$, and $m'\psi = 10 \pm 2$, then $\log K^{RD,Na}_{T} = -0.5 \pm 1.7$. Any increase in anion binding will increase the calculated value of $K^{RD,Na}_{T}$. A reduction in the pK of the titrating groups increases the calculated value of $K^{RD,Na}_{T}$ slightly. On the basis of data of Latt and Sober (1967) on the interactions of oligolysines with poly[r(A)]·poly[r(U)] and poly[r(I)·poly[r(C)], Record et al. (1976) concluded that each ionic interaction between a lysine and a phosphate contributes −0.13 to $\log K_T$. If $m' = 12 \pm 2$ ionic interactions (the maximum value), then these will contribute approximately -1.6 ± 0.3 to $\log K^{RD,Na}_{T}$. This number is within the range of those calculated above for what appear to be reasonable assumptions regarding protonation and anion binding. A consistent picture of the RD interaction from our results is therefore that 12 ± 2 ionic interactions are formed, that two groups on repressor protonate in the interaction with the DNA, that 11 ± 2 Na^+ ions and 0-1 Cl^- ions are released, and that the interaction is driven entirely by the free energy of ion release. Within the framework of this model calculation, $\log K^{RD,Na}_{T}$ can be accounted for entirely by ionic interaction contributions, and no additional contributions to the free energy of interaction from hydrophobic effects or other noncovalent interactions need be sought. (We stress again that this forms a reasonable, self-consistent, and simple picture, but that the semiquantitative nature of the calculations makes it impossible to exclude other alternatives.) A comparison with the thermodynamics of the repressor-operator interaction will be made in the following paper (Record et al., 1977).

(2) *The Thermodynamic Parameters of the RD Interaction in $MgCl_2$.* Subject to the above assumptions, $\log K^{RD,Mg}_{T}$ can be evaluated from the data in Figure 3. We take $\log K^{RD}_{obsd} = 5.65$ at 0.01 M $MgCl_2$, pH 8, and assume that 6 ± 1 ions are released in the RD interaction. Then if $a = 0$, $r = 2$, and $K_H = 1.6 \times 10^7\ M^{-1}$, we obtain $\log K^{RD,Mg}_{T} = -4.6 \pm 2.0$. If $a = 1$, $K_X = 250\ M^{-1}$, $r = 2$, $K_H = 1.6 \times 10^7\ M^{-1}$, and 5 ± 1 Mg^{2+} ions are released, then $\log K^{RD,Mg}_{T} = -1.9 \pm 2.0$.

For any set of parameters, $\log K^{RD,Mg}_{T} < \log K^{RD,Na}_{T}$. If $a = 0$, then $\log K^{RD,Na}_{T} - \log K^{RD,Mg}_{T} = 1.7$. If $a = 1$, then $\log K^{RD,Na}_{T} - \log K^{RD,Mg}_{T} = 1.4$. The uncertainty in the difference is substantially less than the uncertainties cited for the individual values of $\log K^{RD}_{T}$, as long as the same set of parameters is used in both calculations. From eq 21, the theoretical value of this difference is $(m'/2)\log K^{Mg}_{T}$. We estimate $\log K^{Mg}_{T}$ to be in the range 0.3-0.6 from competition experiments described in the following paper (Record et al., 1977). A value of $\log K^{Mg}_{T}$ at the lower end of this range would give good agreement between theory and experiment. To refine these calculations involving magnitudes of the binding constants rather than their derivatives, substantially higher accuracy is required than that provided by our experimental method.

(3) *The Role of Counterion Release as a Driving Force for an Association Reaction.* Record et al. (1976a) demonstrated that the interactions of oligolysines and other model ligands with nucleic acids are driven entirely by the entropic effect of counterion release. The thermodynamic association constants K_T for these reactions are substantially less than unity ($\log K_T < 0$). The reactions are therefore unfavorable at the 1 M standard state condition; association occurs at lower salt concentrations because of the entropic contribution of counterion release.

The same conclusion appears warranted for the nonspecific repressor-DNA interaction. Our best estimates of $\log K^{RD}_{T}$ in NaCl and $MgCl_2$ are discussed in the above sections: $\log K^{RD}_{T} < 0$ in both cases. Consequently the RD interaction appears to be unfavorable in the 1 M standard state and occurs to a measurable extent only at low salt concentrations.

A significant difference between the RD interaction and the model ligands previously considered (Record et al., 1976) is the requirement for protonation at two sites on repressor in order for association to occur. In monovalent salt, the following set of coupled reactions must occur:

$$R_{0,0} + H^+ \overset{2K_H}{\rightleftharpoons} R_{1,0} \qquad (22)$$

$$R_{1,0} + H^+ \overset{K_H/2}{\rightleftharpoons} R_{2,0} \qquad (23)$$

$$R_{2,0} + D_0 \overset{K^{RD,Na}_{T}}{\rightleftharpoons} RD + (11 \pm 2)Na^+ \qquad (24)$$

(The system of subscripts is the same as that defined in connection with eq 4. Equation 24 contains the assumption that all ions released in the observed reaction (cf. Figures 1-2) are Na^+ ions, as discussed above.) A reduction in the NaCl concentration shifts reaction 24 to the right, increasing the concentration of the RD complex. The coupled protonation reactions, for which the free energy change is positive at pH 7.7-8.4 (the range investigated), are driven by reaction 24. The entropic effect of release of counterions into a sufficiently dilute ionic solution drives the entire reaction sequence.

The enthalpy change in reaction 24 is likely to be small, unless the RD complex involves specific interactions between the protein and bases on the DNA. Previous evidence on this point is contradictory (cf. Bourgeois and Pfahl, 1976). The enthalpy change in the interaction of Mg^{2+} with RNA is small (Krakauer, 1971). The enthalpy of interaction of pentalysine with T7 DNA at pH 6, where all groups are protonated, is essentially zero (Lohman and Record, unpublished). The origin of the observed negative enthalpy change in the RD interaction lies in the protonation steps (eq 22 and 23). It is more difficult to protonate either histidine or α-NH_2 groups at high temperature; hence the ΔH of protonation is negative. In the ab-

sence of other enthalpic contributions, the ΔH of protonation is the observed ΔH of the reaction. Nonetheless the fundamental driving force for the coupled reaction sequence is counterion release. In the absence of competing reactions (aggregation, conformational changes), the observed enthalpy change in the RD interaction should decrease with decreasing pH and become negligible when repressor exists in the correct state of protonation for DNA binding.

(4) *Biological Implications of These Results.* Both von Hippel et al. (1974, 1975) and Lin and Riggs (1975) have concluded that the nonspecific binding of *lac* repressor is a necessary factor in the control of expression of the *lac* operon. Pfahl (1976) invokes the tight binding of the X86 mutant repressor to non-operator DNA sites, in order to explain the anomalous effect of low inducer concentrations in *E. coli* cells carrying the X86 mutation. The experiments reported here and those of Revzin and von Hippel (1977) indicate that the observed nonspecific association constant is extremely sensitive to ion concentration changes in the physiological region. (The presence of Mg^{2+} and Na^+ ions together, in roughly their in vivo ratio, reduces these variations of K^{RD}_{obsd} somewhat (Record et al., 1977).) Moreover we show that the binding of one repressor molecule to DNA releases a large number of ions (11 ± 2 monovalent ions) and requires the uptake of two protons.

It seems reasonable to suggest that the involvement of small ions in protein–nucleic acid interactions is of general biological relevance. Different processes could be differentially sensitive to changes in ion concentration (e.g., because the interactions involve the release of different numbers of ions). Then one can envisage such possibilities as the fine tuning of genetic processes by variations in ion concentrations or pH, or also drastic alterations in the pattern of gene expression due to large changes in ion concentrations (e.g., upon virus infection; Carrasco and Smith, 1976), or pH (e.g., effects of pH of the medium on the sporulation of yeast cells; Wejksnora and Haber, 1976).

Inasmuch as the steep dependences on ionic environment of the observed binding constants of protein–nucleic acid interactions are a general phenomenon, it is necessary to have an accurate knowledge of the ionic conditions inside the cell before results obtained in vitro can be related to the situation in vivo. Estimates of the in vivo magnitudes of K^{RD}_{obsd} and K^{RO}_{obsd} are compared in the following paper (Record et al., 1977).

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