Stability of a Lac Repressor Mediated "Looped Complex"†

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ABSTRACT: The quantitation of the stability of a protein-mediated "looped complex" of the Lac repressor and DNA containing two protein-binding sites whose centers of symmetry are separated by 11 helical turns (114 bp) was accomplished by footprint and gel mobility-shift titration techniques. Lac repressor binding to this DNA was only moderately cooperative; a cooperative free energy of −1.0 kcal/mol was calculated in a model-independent fashion from the individual-site loading energies obtained from the footprint titration studies. In order to partition the cooperative binding energy into components representing the dimer–tetramer association of Lac repressor and the cyclization probability of the intervening DNA, advantage was taken of the presence of experimental measures that were in proportion to the concentration of the looped complex present in solution. One measure was the DNase I hypersensitivity observed in footprint titrations in bands located between the two binding sites. The second measure resulted from the electrophoretic resolution in the gel mobility-shift titrations of the band representing the doubly liganded "tandem complex" from the band representing the singly liganded complexes, including the looped complex. Analysis of the footprint and mobility-shift titration data utilizing this additional information showed that approximately 65% of the molecules present in solution are looped complexes at pH 7.0, 100 mM KCl, and 20 °C when the binding sites on the DNA are saturated with protein. Reconciliation of the observed low binding cooperativity and the high proportion of looped complexes could only be obtained when the titration data were analyzed by a model in which Lac repressor tetramers dissociate into dimers in solution. The proportion of looped complexes present in solution is highly dependent on the dimer–tetramer association constant, ΔG_{tet}. This result is consistent with the determination by high-pressure fluorescence techniques that Lac repressor tetramers dissociate with an association free energy comparable to their DNA-binding free energies [Royer, C. A., Chakerian, A. E., & Matthews, K. S. (1990) *Biochemistry 29*, 4959–4966]. However, when the value of ΔG_{tet} of −10.6 kcal/mol (at 20 °C) reported by Royer et al. (1990) is assumed, the titration data demand that tetramers bind DNA with much greater affinity than dimers: a result inconsistent with the destabilization of tetramers by the operator observed in the dimer–tetramer dissociation studies. Analysis of the titration data subject to the assumption that the DNA-binding affinity of dimers and tetramers are identical yields a value of free energy of dimer–tetramer association, ΔG_{tet}, of −13.2 ± 0.5 kcal/mol and a value of the "cyclization free energy", ΔG_j, of +12.2 ± 0.2 kcal/mol. Since the DNA-binding free energies of the Lac repressor to the O_E^L and O_1^L binding sites are −13.8 and −13.7 ± 0.2 kcal/mol, respectively, the result is a looped complex that is only 10–15-fold more stable than the DNA–tetramer complex containing only a single protein–DNA interaction. The cyclization probability, or "*j* factor" [Shore, D., & Baldwin, R. L. (1983) *J. Mol. Biol. 170*, 957–981], calculated from ΔG_j is approximately 0.8×10^{-9} for the intervening DNA in the looped complex. This probability corresponds to that predicted for the cyclization of a DNA fragment of approximately 16 helical turns (167 bp). This value is comparable, to a first approximation, with that expected for the cyclization probability of free DNA when the length of the bridging protein is included in the calculation of the size of the loop.

Communication between control elements widely separated on DNA is an important mechanism in the regulation of cellular processes such as transcription initiation, recombination, and DNA replication in both prokaryotes and eukaryotes. Although a wide variety of mechanisms may exist by which these control elements exert their regulatory effects [cf. Ptashne (1986)], one mechanism that appears to be important in both prokaryotic and eukaryotic systems is the direct interaction of proteins bound to widely separated sites on the DNA to form "DNA loops". Protein-mediated "looped complexes" have a variety of functions. A looped complex may serve to bring an "activation domain" into contact with a target enzyme [cf. Ptashne (1986); and Wendel et al. (1990)], increase the local concentration of a regulatory protein [cf. Mossing and Record (1986); Bellomy et al. (1988)], facilitate transfer of a protein from one region of DNA to another (Von Hippel & Berg, 1989), create a nucleoprotein complex that is the appropriate substrate for an enzyme [cf. Echols (1986); and Moitoso de Vargas (1989)], or create a nucleoprotein complex that inhibits an enzyme. An example of the last category is negative regulation of transcription initiation [cf. Adhya (1989); and Gralla (1989)].

Two systems in which DNA looping appears to be a key component in the regulation of transcription initiation are the *gal* (Majumdar & Adhya, 1984; Haber & Adhya, 1988; Mandal et al., 1990) and *lac* operons of *Escherichia coli* (Borowiec et al., 1987; Eismann et al., 1987; Hsieh et al., 1987; Mossing & Record, 1986). Despite the high degree of homology between the Gal and Lac repressors (von Wilcken-Bergmann & Muller-Hill, 1982), there is a significant difference in the role of DNA looping in regulating the activity of the *gal* and *lac* operons. In the *lac* operon, the rate of transcription initiation is primarily dependent upon the oc-

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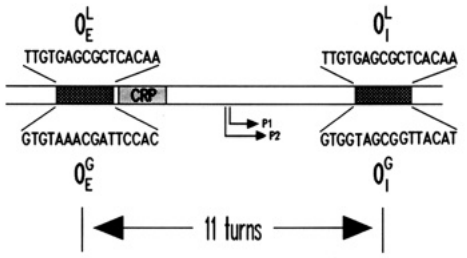

FIGURE 1: Schematic representation of the *gal* operon showing the DNA sequence following conversion of the O_E and O_I operators from *gal* to *lac* recognition sequences (O_E^L and O_I^L). Note that the spacing between the two operators is conserved. The diagram is not to scale.

cupancy of the primary-operator site by the Lac repressor. The occupancy of the primary operator is increased by the simultaneous interaction of a Lac repressor tetramer with the primary and pseudo-operators. The presence of Lac repressor mediated DNA loops has been demonstrated both in vivo and in vitro [cf. Whitson et al. (1986); Borowiec et al. (1987); Kramer et al. (1987); and Eismann and Muller-Hill (1990)]. In addition, aspects of the thermodynamics of the formation of Lac repressor mediated looped complexes, in vivo, have been described by Record and co-workers (Mossing & Record, 1986; Bellomy et al., 1988).

In contrast, neither the O_E nor the O_I operators of the *gal* operon overlap the promoter (Figure 1). In vivo transcription studies indicate that although both O_E and O_I are required for negative regulation, the simple occupancy of O_E and O_I by a protein is not sufficient for repression (Haber & Adhya, 1988; Adhya, 1989; Mandal et al., 1990). By converting the sequences of O_E and O_I from *gal* to *lac* recognition sequences, Haber and Adhya (1988) demonstrated that the *gal* operon could be negatively regulated by the Lac repressor but not by a Lac repressor mutant capable of association only to dimers (Mandal et al., 1990). Thus, it appears that a protein-mediated looped complex, rather than a unique property of Gal repressor, can inhibit transcription initiation from the *gal* promoter. However, in vitro analysis by electron microscopy (Mandal et al., 1990) and DNA-binding titration assays (Brenowitz et al., 1990) have not shown the presence of Gal repressor mediated looped complexes.

Lac repressor mediated looped complexes result from the ability of the protein tetramer to act as a bidentate ligand. It has generally been assumed that the Lac repressor formed stable tetramers in solution. However, the dimer–tetramer association free energy, ΔG_{tet}, for the Lac repressor was recently shown to be −10.6 kcal/mol at 20 °C (Royer et al., 1990). This value of ΔG_{tet} would result in the Lac repressor existing predominantly as a dimer in the concentration ranges under which this protein has been investigated, in vitro [cf. Ha et al. (1989); and this paper]. A general description of the binding configurations of Lac repressor dimers and tetramers with DNA containing two separated binding sites is depicted in the thermodynamic linkage scheme shown in Figure 2. It is clear from this scheme that dimer–tetramer association is an important component looped complex formation. An additional factor to be considered is the possibility of linkage between dimer–tetramer association and DNA-binding affinity. For these reasons, quantitative methods to directly measure the thermodynamics of protein-mediated looped complexes, in vitro, have been developed.

The system chosen for these investigations is a hybrid of the *gal* and *lac* systems. The O_E and O_I operators of the *gal* control region were mutated to identical sequences recognized with high affinity by the Lac repressor (Haber & Adhya, 1988;

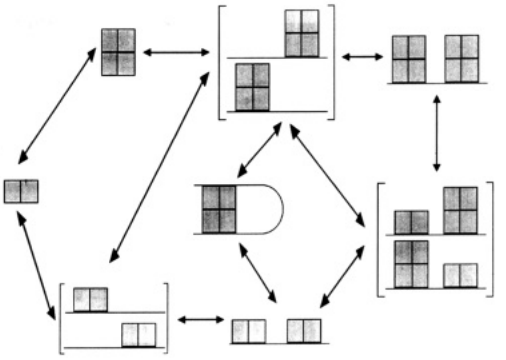

FIGURE 2: Schematic representation of the configurations present when thermodynamic linkage exists between Lac repressor self-association and DNA binding to a two-site operator. The assumption made in this scheme is that the Lac repressor does not dissociate into monomers (Royer et al., 1990). For clarity, the restricted case of two identical binding sites is shown. The asymmetric complexes are depicted in the two energetically equivalent configurations.

Figure 1). This simple hybrid system provides a useful model for quantitative analysis of looped complexes for several reasons: (1) Lac repressor is capable of regulating this modified *gal* operon (Haber & Adhya, 1988; Mandal et al., 1990). (2) The *gal* operator sites are nominally "in phase" with each other, i.e., on the same side of the helix, eliminating, at least to a first approximation, the need to consider torsion of the DNA in evaluating the cyclization probabilities [cf. Bellomy et al. (1988) and references therein]. (3) Two high-affinity identical protein-binding sites provide the simplest model system capable of forming a DNA loop. (4) The in vivo studies by Haber and Adhya (1988) suggest that a looped complex is the negative regulatory element in the *gal* operon rather than the binding of a particular protein. (5) An understanding of the ability of *gal* DNA to form a DNA loop may prove helpful in understanding the absence of Gal repressor mediated DNA loops in vitro.

Materials and Methods

DNA Operators. The construction of operators in which the *gal* recognition sequences (O_E^G and O_I^G) were altered to *lac* recognition sequences (O_E^L and O_I^L) has been described (Haber & Adhya, 1988). The resultant *lac* recognition sequence is shown in Figure 1. Linear DNA restriction fragments of 635 bp labeled with ^{32}P at only one end were generated by cleavage of the plasmids with *Eco*RI, labeling with ^{32}P, and a secondary cleavage with *Hin*dIII. The fragments were purified by agarose gel electrophoresis and electroelution. Detailed protocols describing these procedures have been published (Brenowitz et al., 1986a; Brenowitz & Senear, 1989). These linear restriction fragments were used in all DNA-binding experiments.

Purification and DNA-Binding Activity of the Lac Repressor. The Lac repressor was purified as described in Brenowitz et al. (1991). The DNA-binding activity of the Lac repressor preparation used in these studies is assumed to be 100%. This assumption was made because several-fold variability in activity measurements has been observed for both Gal and Lac repressors (unpublished data). Heyduk and Lee (1990) have reported lower apparent DNA-binding activities for cAMP receptor protein (CRP) when activity is measured by utilizing DNA restriction fragments compared with oligonucleotides. Studies to resolve this issue for the Gal and Lac repressors are in progress.

Calculation of Free Dimer and Tetramer Concentrations. The concentrations of Lac repressor dimers and tetramers were calculated by assuming that 2[dimer] $\rightleftarrows$ [tetramer] is the sole

Table I: Free Energies of the Microscopic Configurations Describing Lac Repressor Binding to a Two-Site Operator in Which a Protein-Mediated "DNA-Loop" Can Form

species	O_E^L		O_I^L	free energy terms[a]
1				reference state
2	D			ΔG_E^d
3			D	ΔG_I^d
4	D		D	$\Delta G_E^d + \Delta G_I^d$
5	T			ΔG_E^t
6			T	ΔG_I^t
7	T		T	$\Delta G_E^t + \Delta G_I^t$
8		—T—		$\Delta G_E^t + \Delta G_I^t + \Delta G_j$
9	D		T	$\Delta G_E^d + \Delta G_I^t$
10	T		D	$\Delta G_E^t + \Delta G_I^d$

[a] Free energy terms are defined in the text and are expressed in kcal/mol (see Materials and Methods). D represents protein dimers, and T represents protein tetramers. The free concentration of tetramers is related to the free concentration of dimers by the expression $[T] = k_{tet}[D]^2$.

equilibria necessary to describe the self-association of the protein in the concentration range used in these studies (Royer et al., 1990). The assumption was also made that $[repressor]_{total} \approx [repressor]_{free}$ since the concentration of operator sites in the equilibrium mixtures ($\leq$5 pM) is low relative to the equilibrium DNA-binding constants.

DNA-Binding Assays. The quantitative DNase I footprint titration experiments were conducted essentially as described in Brenowitz et al. (1986a,b) and Brenowitz and Senear (1989). The degree of nicking by DNase I was carefully controlled so that, on the average, each DNA molecule that was nicked, was nicked only once (Brenowitz et al., 1986a). This control minimized the possibility that the DNase I exposure perturbed the repressor–DNA equilibrium by, for example, compromising the integrity of the double-stranded DNA located between O_E^L and O_I^L by the introduction of multiple single-stranded nicks.

The quantitative gel mobility-shift assays were conducted as described in Brenowitz et al. (1990, 1991). The only differences in the mobility-shift protocols used herein were the use of 4% polyacrylamide gels and electrophoresis times of 3.5 h. The assay buffer in which protein and DNA were equilibrated contained 25 mM Bis-Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 50 μg/mL BSA, 2 μg/mL calf thymus DNA, and 100 mM KCl titrated to pH 7 with HCl at 20 °C. In addition, 3% glycerol was present in the assay buffer used in the gel mobility-shift experiments to facilitate sample loading. All experiments were conducted at 20 °C.

Digital representations of both footprint and mobility-shift titration autoradiograms were obtained using a microcomputer-based video-densitometer (Reiner & Brenowitz, 1991). Densitometric analysis was conducted by using a microcomputer implementation (M. Brenowitz, P. Reiner, and B. Turner, unpublished computer programs) of the computer software originally described in Brenowitz et al. (1986a,b).

Statistical Mechanical Model of Binding. A general model describing the interaction of monovalent dimers and bidentate tetramers to a two-site operator is shown in Table I. Because of the low concentration of the DNA in the equilibrium mixtures, "sandwich complexes" in which a single tetramer bridges two DNA molecules [cf. Kramer et al. (1987)], are deemed negligible and not included in the model. ΔG_i^d and ΔG_i^t represent the "intrinsic" binding free energies, i.e., the binding of dimers and tetramers, respectively, to one site in the absence of binding to others. ΔG_{tet} represents the self-association of *unliganded* dimers to tetramers.

ΔG_j represents the "free energy of cyclization", which is proportional to the "cyclization probability" (or "j factor") of the DNA within the protein–DNA complex. Cyclization probability, which has been utilized as a measure of the flexibility of DNA, is defined as the ratio K_c/K_a where K_c is the equilibrium constant for cyclization of a linear molecule and K_a is the bimolecular equilibrium constant for the association of two distinguishable half molecules [cf. Shore and Baldwin (1983)]. An equivalent expression for j, appropriate to the studies conducted herein, is the ratio of equilibrium constants describing the looped and tandem complexes (Table I, species 8 and 7, respectively). This expression is $j_{tet} = k_E k_I k_j / k_E k_I = k_j$, where the product of the equilibrium binding constants have been substituted for the sum of the Gibbs free energies by utilizing the well-known expression, $\Delta G_i = -RT \ln k_i$. The ΔG_j term includes both the entropic and energetic contributions to the energy involved in juxtaposing the second binding site in the looped complex (see Discussion).

This model rests on several assumptions: (1) Lac repressor dimers and tetramers are in equilibrium and are the only species present in solution [cf. Royer et al. (1990)]. (2) The dimer possesses one, and the tetramer, two DNA-binding sites. The constraint that the two sites within the tetramer are equal and independent is used in most of the analysis presented. A direct analysis of cooperativity within the Lac tetramer is beyond the scope of this paper. (3) Formation of looped complexes occurs via association of two bound dimers or via the binding of a single tetramer. The cyclization probability of the DNA, ΔG_j, to form the bidentate complex is the same whether the looped complex is formed via association of two bound dimers or binding of a single tetramer. These assumptions are based on the thermodynamic principle that the Gibbs free energy is a state function and hence independent of the path used to attain that state. (4) "Tandem complexes" composed of two bound tetramers or one bound dimer and one bound tetramer cannot mediate formation of a looped complex.

Analysis of Footprint Titrations. Individual-site binding equations describing the fractional saturation of O_I^L and O_E^L can be constructed by considering the relative probability of each configuration described by the model shown in Table I (Ackers et al., 1982, 1983). The probability of an operator in a given configuration can be written

$$P_s = \frac{\exp(-\Delta G_s/RT)[P]^j}{\sum_{sj}\exp(-\Delta G_s/RT)[P]^j} \quad (1)$$

where P_s is the probability of a species s (Table I), ΔG_s is the free energy of species, s, R is the gas constant, T is the temperature in degrees Kelvin, [P] represents the free protein concentration, and j is the stoichiometry of the binding protein.

The equations relating the fractional probability of the configurations with the fractional saturation of a binding site are

$$\bar{Y}_E = f_2 + f_4 + f_5 + f_7 + f_8 + f_9 + f_{10} \quad (2)$$

and

$$\bar{Y}_I = f_3 + f_4 + f_6 + f_7 + f_8 + f_9 + f_{10} \quad (3)$$

where Y_i represents the fractional saturation of the binding at the indicated site and f_j's represent the fractional probabilities of the configurations shown in Table I (Ackers et al., 1982, 1983). Expanding these expressions by using the free energy terms shown in Table I, and collecting terms, yields the binding polynomial

$$Z = 1 + (k_I^d + k_E^d)[D] + (k_I^t + k_E^t + k_I^t k_E^t k_j)[2T] + k_I^d k_E^d[D]^2 + k_I^t k_E^t[2T]^2 + (k_I^t k_E^d + k_I^d k_E^t)[D][2T] \quad (4)$$

and the individual-site binding equations for sites O_I^L and O_E^L

$$\bar{Y}_E = \frac{k_E^d[D] + (k_E^t + k_I^t k_E^t k_j)[2T] + k_I^d k_E^d[D]^2}{Z} + \frac{k_I^t k_E^t[2T]^2 + (k_I^t k_E^d + k_I^d k_E^t)[D][2T]}{Z} \quad (5)$$

and

$$\bar{Y}_I = \frac{k_I^d[D] + (k_I^t + k_I^t k_E^t k_j)[2T] + k_I^d k_E^d[D]^2}{Z} + \frac{k_I^t k_E^t[2T]^2 + (k_I^t k_E^d + k_I^d k_E^t)[D][2T]}{Z} \quad (6)$$

where the microscopic equilibrium constants have been substituted for the Gibbs free energies by using the relation $\Delta G = -RT \ln k$. [D] and [T] represent the free concentrations of repressor dimer and tetramer, respectively. The statistical factor of 2 preceding [T] in all configurations binding the tetramer is due to each tetramer possessing two binding sites for DNA.

On footprint titration autoradiograms, bands between O_E^L and O_I^L became hypersensitive to DNase I upon the titration of the O_E^L/O_I^L operator with the Lac repressor. These bands are believed to be quantitative indicators of the concentration of looped complex present in solution (see Results). These data are described by the binding equation

$$\bar{Y}_{loop} = f_8 \quad (7)$$

$$\bar{Y}_{loop} = \frac{k_I^t k_E^t k_j[2T]}{Z} \quad (8)$$

For operators in which either O_E or O_I were mutated to prevent repressor binding, referred to as "reduced-valency mutants" (Ackers et al., 1982), the *individual-site* binding equations reduce to

$$\bar{Y}_j = \frac{k_j^d[D] + k_j^t[2T]}{1 + k_j^d[D] + k_j^t[2T]} \quad (9)$$

where j refers to either site O_E^L or site O_I^L.

Conversion of the transition curves determined from the footprint titration autoradiograms to binding curves that represent fractional saturation must be accomplished. This was done by scaling the data with

$$\bar{Y} = m \cdot \bar{Y}_{app} + b \quad (10)$$

where $\bar{Y}_{app}$ represents the normalized integrated optical density of a site determined from an autoradiogram and $m = 1/(u-l)$ and $b = l/(l-u)$, u and l being the best-fit upper and lower endpoints, respectively (Brenowitz et al., 1986a; Brenowitz & Senear, 1989). For this technique to be valid, it is essential that both the upper and the lower titration plateaus are clearly defined. This scaling procdure was also applied to the analysis of $\bar{Y}_{loop}$ from the DNase I hypersensitivity data.

Analysis of Gel Mobility-Shift Titrations. The gel mobility-shift assay (Fried & Crothers, 1981; Garner & Revzin, 1981) measures the fraction of DNA molecules with i ligands bound, where i varies from 0 to the number of binding-sites. In addition, this technique can potentially differentiate molecules with identical ligation states but different configurations [cf. Kramer et al. (1987)]. Separate configurations of bound dimers and tetramers could not be distinguished under our experimental conditions (see Results and Discussion). The binding curves that measure the fractions of molecules, Θ_i, that are either unliganded, singly liganded, singly liganded in a looped complex, or doubly liganded can be written by following the principles used to construct the footprint titration binding expressions (Senear et al., 1986). These expressions are

$$\Theta_0 = \frac{1}{Z} \quad (11)$$

$$\Theta_1 = \frac{(k_E^d + k_I^d)[D] + (k_E^t + k_I^t)[2T]}{Z} \quad (12)$$

$$\Theta_{loop} = \frac{k_I^t k_E^t k_j[2T]}{Z} \quad (13)$$

$$\Theta_2 = \frac{(k_E^d k_I^d)[D]^2 + (k_E^t k_I^t)[2T]^2 + (k_I^t k_E^d + k_I^d k_E^t)[D][2T]}{Z} \quad (14)$$

respectively. As described for the footprint titration experiment, the equations reduce to

$$\Theta_0 = \frac{1}{1 + k_j^d[D] + k_j^t[2T]} \quad (15)$$

and

$$\Theta_1 = \frac{k_j^d[D] + k_j^t[2T]}{1 + k_j^d[D] + k_j^t[2T]} \quad (16)$$

for analysis of single-site reduced-valency operators where Θ_0 and Θ_1 represent the fractions of unliganded and singly liganded molecules, respectively, and j refers to either site O_I^L or site O_E^L.

The observation that the singly liganded and the looped complex occupy the same electrophoretic band (Cann, 1990; see Results) was incorporated into the analysis of the mobility-shift titrations by combining eqs 12 and 13 into a composite binding expression:

$$\Theta_{1,loop} = \Theta_1 + \Theta_{loop} \quad (17)$$

The fraction of molecules in each configuration was calculated at each protein concentration as $\Theta_{lane} = D_i/D_{total}$ where D_i is the density of band i and D_{total} is the sum of the densities of all the bands in a lane (Brenowitz et al., 1990). Numerical scaling of the titration curve endpoints (eq 10) did not significantly alter the results obtained from the gel mobility-shift data (unpublished analysis).

All data were fit to the appropriate equations by methods of nonlinear least-squares parameter estimation to determine the best-fit values of the parameters, their 65% confidence limits, and the variance of the fit (Johnson & Frasier, 1985).

Results

Footprint Titration Analysis. Individual-site binding curves were determined from the footprint titrations for each site of the two-site O_E^L/O_I^L operator (Figure 3A) and the two reduced-valency single-site operators, O_E^L/O_I^G (Figure 3C) and O_E^G/O_I^L (Figure 3D). Note that two binding curves, one for O_E^L and one for O_I^L, are obtained for the titrations of the O_E^L/O_I^L operator (Figure 3A, open and closed symbols, respectively). The concentration range of the titrations spanned almost six orders of magnitude, sufficient to ensure accurate numerical determination of the upper and lower titration endpoints (eq 10).

The "individual-site loading energy", ΔG^ℓ, of a site represents the chemical work required to bind a protein to that site and can be determined from the median ligand activity of the titration curves (Ackers et al., 1983; Senear & Ackers, 1990).

Table II: Loading Free Energies of Binding Lac Repressor to O_E^L/O_I^L, O_E^L/O_I^G, and O_E^G/O_I^L Operators[a]

operator	ΔG_E^{ℓ}	ΔG_I^{ℓ}
O_E^L/O_I^L	−14.4	−14.1
O_E^L/O_I^G	−13.8	
O_E^G/O_I^L		−13.7

[a] The median ligand activity of each titration curve was estimated from the midpoints of the best-fit Langmuir binding isotherm. Free energies are expressed in kcal/(mole of tetramer).

The values of ΔG^{ℓ} that were calculated for each site from the simultaneous titration of O_E^L/O_I^L with the Lac repressor are shown in the top line of Table II. Similar calculations were conducted for titrations of Lac repressor binding to the two single-site operators, O_E^L/O_I^G and O_E^G/O_I^L (Table II, lines 2 and 3). Note that the values of ΔG^{ℓ} for each site of the two-site O_E^L/O_I^L operator are greater than those of the two single-site operators. A "model-independent" estimation of the cooperativity of Lac binding to O_E^L/O_I^L can be obtained from the difference between the loading energies obtained from binding Lac repressor to O_E^L and O_I^L simultaneously and binding to each site individually (Ackers et al., 1983). An assumption made in this analysis is that base-pair substitutions in one binding site do not affect the intrinsic binding of protein to the other binding site [see Brenowitz et al. (1986a) and Senear and Ackers (1990) for discussions of this issue]. Subtraction of the loading energies of the single-site reduced-valency operators (O_E^L/O_I^G and O_E^G/O_I^L) from those of the two-site operator (O_E^L/O_I^L) yields values of the "cooperative loading energy" for each site of −0.6 and −0.4 kcal/mol for O_E and O_I, respectively, or a total cooperative loading energy of −1.0 kcal/mol. The approximately equal partitioning of the cooperative energy between the two sites is consistent with the two-sites possessing equal intrinsic binding affinities for the Lac repressor (Ackers et al., 1983).

The O_E^L/O_I^L operator was first analyzed by minimization of eqs 5 and 6 to the titration curves measured for O_E^L and O_I^L, respectively. The titration curves are almost completely insensitive to pairs of values of ΔG_j and ΔG_{tet} due to the high numerical correlation between the two energies (Table III). This correlation between parameters can be reduced by the simultaneous analysis of the titrations of the O_E^L/O_I^L operator (eqs 5 and 6) with the titrations of the single-site O_E^G/O_I^L and O_E^L/O_I^G operators (eq 9). In this analysis, values of the energies are obtained that describe a common minima for all four titration curves. Values of ΔG_E, ΔG_I, and ΔG_j were obtained (subject to the assumption that $\Delta G_E^d = \Delta G_E^t$ and $\Delta G_I^d = \Delta G_I^t$) when ΔG_{tet} was set equal to −25 kcal/mol (a condition describing a nondissociable tetramer) and to two values describing increasing dissociation of tetramer to dimer (Table III, bottom three lines). This analysis yielded slight evidence that a coupled transition was favored; slightly smaller variances were obtained when ΔG_{tet} was set equal to −13.0 or

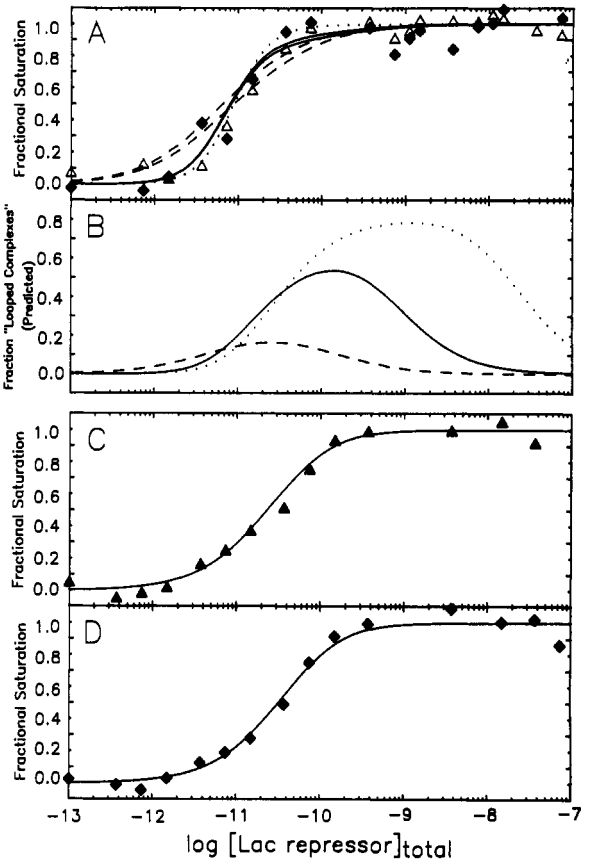

FIGURE 3: Individual-site binding titrations of the Lac repressor with O_E^L/O_I^L (A), O_E^L/O_I^G (C), and O_E^G/O_I^L (D) operators. In C and D only O_E^L or O_I^L, respectively, are able to bind the Lac repressor (see Figure 1). Titration data points for O_E^L and O_I^L are represented by triangles and diamonds, respectively. Open and solid symbols are used in panel A in order to clearly denote the two individual-site binding curves obtained upon titration of the O_E^L/O_I^L operator with the Lac repressor. The curves were calcualted with ΔG_{tet} = −25 (dashed lines), −13.0 (solid lines), and −10.6 kcal/mol (dotted lines) from the best-fit values shown in Table III with the assumption that the DNA-binding affinity of dimers and tetramers are identical. The curves shown in B represent the predicted concentrations of looped complexes (eq 8) at each of the values of ΔG_{tet}.

Table III: Simultaneous Analysis of the Binding of the Lac Repressor to the O_E^L/O_I^L, O_I^G/O_E^L, and O_I^L/O_E^G Operators[a]

operator	ΔG_E	ΔG_I	ΔG_j	ΔG_{tet}	s^b
O_E^G/O_I^L		−13.7 ± 0.2		(−25)[c]	0.060
O_E^L/O_I^G	−13.8 ± 0.2			(−25)	0.056
O_E^L/O_I^L	−12.0 ± 0.7	−11.9 ± 0.8	(10)	(−25)	0.097
	−13.0 ± 0.2	−12.9 ± 0.5	(12)	(−25)	0.103
	−13.7 ± 0.2	−13.5 ± 0.3	(14)	(−25)	0.107
	−13.6 ± 0.5	−13.2 ± 0.2	(10)	(−10.6)	0.096
	−13.8 ± 0.3	−13.6 ± 0.4	(12)	(−10.6)	0.104
	−13.7 ± 0.4	−13.9 ± 0.4	(14)	(−10.6)	0.107
Simultaneous Analysis of O_E^L/O_I^L, O_E^G/O_I^L, and O_E^L/O_I^G					
	−13.9 ± 0.3	−13.7 ± 0.3	≥13.7[d]	(−25)	0.089
	−13.7 ± 0.3	−13.6 ± 0.3	12.5 ± 0.6	(−13)	0.084
	−13.7 ± 0.2	−13.6 ± 0.2	10.5 ± 0.5	(−10.6)	0.081

[a] These data were analyzed subject to the constraint that Lac repressor dimers and tetramers have equal DNA-binding affinity, i.e., that $\Delta G_E^d = \Delta G_E^t$ and $\Delta G_I^d = \Delta G_I^t$. [b] Square root of the variance. Bracketed values do not differ significantly. [c] The dimer–tetramer association free energy of −25 represents a nondissociable tetramer, where the proportion of dimer is negligible over the entire titration range. Values in parenthesis were fixed during the nonlinear least-squares analysis. [d] The upper confidence limit of this value was indeterminate.

Table IV: Free Energies Determined from Simultaneous Analysis of Lac Repressor Titrations of the O_E^L/O_E^L, O_E^L/O_I^G, and O_E^G/O_I^L Operators Including the DNase I Hypersensitivity Data Obtained from the O_E^L/O_E^L Titrations[a]

operator	ΔG_E[d]	ΔG_I[d]	ΔG_E^t	ΔG_I^t	ΔG_j	ΔG_{tet}	s[c]
O_E^L/O_I^G			−13.8 ± 0.2			(−25)	0.056 ┐
	−13.7 ± 0.2		−15.3 ± 1.0			(−10.6)[b]	0.056 ┘
O_E^G/O_I^L				−13.7 ± 0.2		(−25)[d]	0.060 ┐
		−13.5 ± 0.2		−15.6 ± 0.9		(−10.6)	0.060 ┘
Simultaneous Analysis of the Three Operators Including the Hypersensitivity Data							
	(= ΔG_E^t)	(= ΔG_I^t)	(−13.8)[e]	(−13.7)[e]	10.9 ± 0.7	(−10.6)	0.319
			(−13.8)[e]	(−13.7)[e]	13.7 ± 0.5	(−25)	0.122
	(= ΔG_E^t)	(= ΔG_I^t)	−13.7 ± 0.2	−13.6 ± 0.2	12.2 ± 0.2	−13.2 ± 0.5	0.088 ┐
	−13.7 ± 0.3	−13.5 ± 0.3	−15.2 ± 0.2	−15.0 ± 0.2	12.8 ± 0.3	(−10.6)	0.090 ┘

[a] Free energies in kcal/mol. [b] The dimer–tetramer association free energy of −10.6 was obtained from Royer et al. (1990). [c] Square root of the variance of the fitted curve. Pairs of results that do not differ significantly are indicated by brackets. [d] The dimer–tetramer association free energy of −25 represents a nondissociable tetramer where the proportion of dimer is negligible over the entire titration range. Values in parentheses were fixed during the nonlinear least-squares analysis. [e] These values were fixed at the values determined for the two reduced-valency operators (see text).

−10.6 kcal/mol rather than to −25 kcal/mol. Note the sharpening of the binding curves predicted for the two conditions describing significant dissociation of tetramers to dimers (Figure 3A, solid and dotted lines), a result consistent with the linkage of the dimer–tetramer equilibrium with the formation of the looped complex. However, the differences among the fits are not significant.

The single-site O_E^L/O_I^G and O_E^G/O_E^L titrations are well-described by isotherms predicted by assuming only a single form of the binding protein (Figure 3C,D). The values of ΔG_E and ΔG_I determined from these curves are identical within experimental error (Table III, lines 1 and 2). Indistinguishable fits were obtained when the Lac repressor was assumed to be all tetrameric and when ΔG_{tet} was assumed to be −10.6 kcal/mol and dimers and tetramers were allowed to have unequal DNA-binding affinities (Table IV, top four lines). This latter result suggests that linked protein–subunit association and DNA-binding equilibria are not issues to be considered when the Lac repressor binds to a single DNA site under these experimental conditions.

The predicted concentration of looped complexes (eq 8) is shown in Figure 3B (dashed line) when it is assumed that the protein exists in solution only as a nondissociable tetramer (ΔG_{tet} = −25 kcal/mol) with equal and independent DNA-binding sites. It is striking that the predicted fraction of molecules in looped complexes reaches a maximum of less than 20%. The displacement of the looped complexes by tandem complexes is driven by mass action at higher protein concentrations. This result is not consistent with the demonstration of high levels of repression of transcription initiation from this operator by the Lac repressor in vivo (Haber & Adhya, 1988). Although DNA supercoiling has been shown to facilitate the formation of DNA loops in several operons, including the *lac* (Borowiec et al., 1987; Whitson et al., 1987a,b; Kramer et al., 1988) and *ara* (Hahn et al., 1986; Lobell & Schleif, 1990) operons, DNA supercoiling does not appear to be required for the repression of transcription at the *gal* promoter (Menzel & Gellert, 1987). Thus, if the looped complex is, in fact, the negative regulatory element of the *gal* promoter (Adhya, 1989), then it would be expected that the looped complex would be the predominant species when the operators are fully occupied by repressor.

An explanation of this discrepancy is found in the coupling of Lac repressor self-association to looped complex formation. Significantly increased fractions of looped complexes are obtained when the association free energy of dimers to tetramers, ΔG_{tet}, is comparable to the intrinsic binding free energies, ΔG_E and ΔG_I. High-pressure fluorescence techniques were recently used to demonstrate that Lac repressor tetramers dissociate to dimers. The value of ΔG_{tet} was determined to be −10.6 kcal/mol at 20 °C (Royer et al., 1990). It is fortuitous that these dissociation studies were carried out under buffer conditions similar to those used herein. When this value of ΔG_{tet} is assumed, the predicted fraction of molecules in looped complexes reaches a broad plateau at approximately 80% (Figure 3B, dotted line). The assumption made in this calculation that the DNA-binding affinity of dimers and tetramers is identical will be discussed below. It is interesting to note that when ΔG_{tet} is assumed to be −10.6 kcal/mol, the Lac repressor is predicted to exist primarily in the form of dimers until the approximate point at which O_E^L and O_I^L are saturated with protein under these experimental conditions.

It is clear from the analyses presented in Table III that additional information is required in order to determine unique values of ΔG_j when subunit association must also be considered. The fact that a number of bands located between O_E^L and O_I^L in the titration autoradiograms of the O_E^L/O_I^L operator become hypersensitive to DNase I upon binding of the Lac repressor (Figure 4; Kramer et al., 1987) presents this additional information. The presence of DNase I hypersensitivity has been observed in systems in which protein-mediated looped complexes have been observed by other techniques [cf. Hochshild and Ptashne (1986); Griffith et al. (1986); Kramer et al. (1987); and Mandal et al. (1990)], suggesting that the hypersensitivity might prove to be a quantitative measure of looped complex concentration. Two observations support this conclusion. First, no hypersensitivity is observed upon Lac repressor binding to the O_E^L/O_I^G and O_E^G/O_I^L single-site operators (data not shown). These operators are incapable of forming a looped complex and only differ from O_E^L/O_I^G by the base-pair substitutions in the protein-binding sites (Haber & Adhya, 1988; Mandal et al., 1990). Second, no DNase I hypersensitivity was observed upon the binding to O_E^L/O_I^L of a Lac repressor mutant incapable of forming tetramers (Brenowitz et al., 1991).

The relative hypersensitivity of the five hypersensitive bands located between O_E^L and O_I^L that are shown in Figure 4 was quantitated (Figure 5). The negative values seen in Figure 5 are the result of the normalization of the integrated optical densities of the bands to the lane containing no repressor (Brenowitz et al., 1986a). In this normalization procedure, the decrease in density observed in DNase I *protection* results in positive values. Since these are *relative* values, these data do not directly yield the fraction of molecules that are looped complexes. The fraction of looped complexes is determined by simultaneously fitting the individual-site data (Figure 3) to eqs 5, 6, and 9 and the relative hypersensitivity data (Figure 5) to eq 8 and by individually scaling all titration endpoints with eq 10. Thus, the concentration of looped complex determined from this analysis is dependent upon the model used

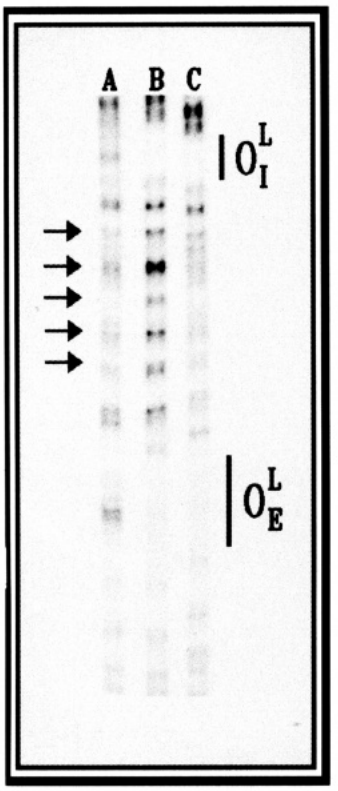

FIGURE 4: Three lanes (lanes 1, 8, and 18) of the autoradiogram of the footprint titration of the O_E^L/O_I^L operator with the Lac repressor that was quantitated in Figure 3A. Lanes A, B, and C contain none, ~0.1 and ~100 nM protein, respectively. Lane B corresponds to the approximate protein concentration where DNase I hypersensitivity reaches a maximum. The arrows indicate the bands whose DNase I hypersensitivity was quantitated. The solid bars indicate the protected regions of O_E^L and O_I^L. The slight misalignment of the bands of is due to nonuniform electrophoresis. The figure was produced from a digital representation of the autoradiogram by using techniques of image analysis.

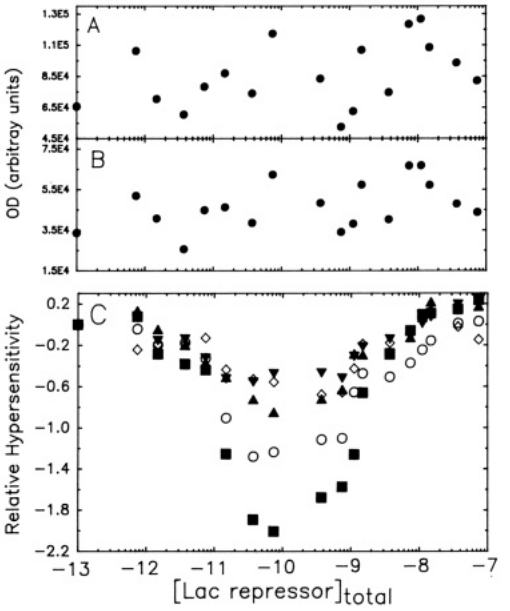

FIGURE 5: (A) Integrated optical density of a "standard block" comprising several bands 5′ to O_E. (B) Integrated optical density of the standard block comprising several bands 3′ to O_I. Note that the variation in the densities is random, demonstrating that the DNase I hypersensitivity observed upon looped complex formation is restricted to the region between O_E and O_I. (C) Quantitation of the relative hypersensitivity of the five bands shown in Figure 4 upon the binding of the Lac repressor. The bands in Figure 4 are represented, from top to bottom, as open circles (band 1), filled triangles (band 2), filled squares (band 3), inverted filled triangles (band 4), and open diamonds (band 5). The integrated optical density of each band was normalized to the density of the band in the lane containing no repressor (Brenowitz et al., 1986a). The negative values reflect the fact that the optical density of hypersensitive bands increases upon protein titration rather than decreases as observed in protected regions.

to analyze the data. The results of an analysis conducted subject to the assumption that dimers have the same DNA-binding affinity as tetramers are shown in Figure 6.

When the titration and hypersensitivity data were fit assuming $\Delta G_{tet} = -25$ kcal/mol or -10.6 kcal/mol, systematic error was observed in the fitted curves to both the hypersensitivity data as well as the two single-site titration data, O_E^L/O_I^G and O_E^G/O_I^L (Table IV, lines 5 and 6). For the purpose of comparison, the values of ΔG_E and ΔG_I were subsequently fixed in the analysis at the values determined from the O_E^L/O_I^G and O_E^G/O_I^L titrations (Table IV, lines 1 and 3) so that all the error in fitted curves would be propagated to the hypersensitive bands (Figure 6A,C). When the data were analyzed with ΔG_E, ΔG_I, ΔG_j, and ΔG_{tet} all floated as fitting parameters, values of ΔG_j of +12.2 and ΔG_{tet} of -13.2 were obtained (Table IV, second to bottom line). All the titration data are well-described over their entire range by the curves predicted by these free energy values [Figure 3 (solid lines) and Figure 6B]. The fraction of looped complex reaches a maximum value of approximately 60% when ΔG_{tet} is equal to -13.2 kcal/mol.

The analysis presented up to this point was conducted subject to the assumption that the DNA-binding affinities of dimers and tetramers are equal. However, Royer et al. (1990) observed that a repressor–operator complex dissociated more easily than the repressor protein alone. If this observation is correct, it is a thermodynamic requirement that the DNA-binding affinity of dimers be *greater* than that of tetramers. In order to test this prediction, the individual-site and DNase I hypersensitive band titration data were analyzed with ΔG_{tet} fixed equal to -10.6 kcal/mol (the value determined by Roger et al.) and with the dimer and tetramer intrinsic binding free energies, ΔG_E^d, ΔG_I^d, ΔG_E^t, and ΔG_I^t, allowed to float independently. These data clearly converged to values where the DNA-binding affinity of dimers of 1.5 kcal/mol *less* than that of tetramers (Table IV). This result is not consistent with both the results that $\Delta G_{tet} = -10.6$ kcal/mol *and* the observation of increased dissociation of the repressor–operator complex. It is not possible to determine both dimer and tetramer DNA-binding affinities and the dimer–tetramer association constant from the binding data due to the numerical correlation of the two energies.

Gel Mobility-Shift Analysis. A limitation to the use of DNase I hypersensitivity as an assay of the concentration of looped complex is the fact that this assay does not directly yield the fraction of molecules. In principle, the gel mobility-shift assay can provide such information if the looped complex migrates as an electrophoretically unique band. Typical mobility-shift titrations of the Lac repressor with the two-site O_E^L/O_I^L and the single-site O_E^L/O_I^G operators are shown in Figure 7, A and B, respectively. In the titration of the single-site operator, the expected transition from a rapid mobility "free DNA" band to a retarded mobility singly liganded complex is observed (Figure 7B). Titration of either O_E^L/O_I^G or O_E^G/O_I^L with the Lac repressor yielded complexes of identical electrophoretic mobility, a result most likely due to the fact that O_E^L and O_I^L are 98 and 212 bp, respectively, from the end of the 635-bp DNA restriction fragment employed in these studies. Thus, the experimental conditions employed in these studies were not ideal to detect the Lac repressor induced

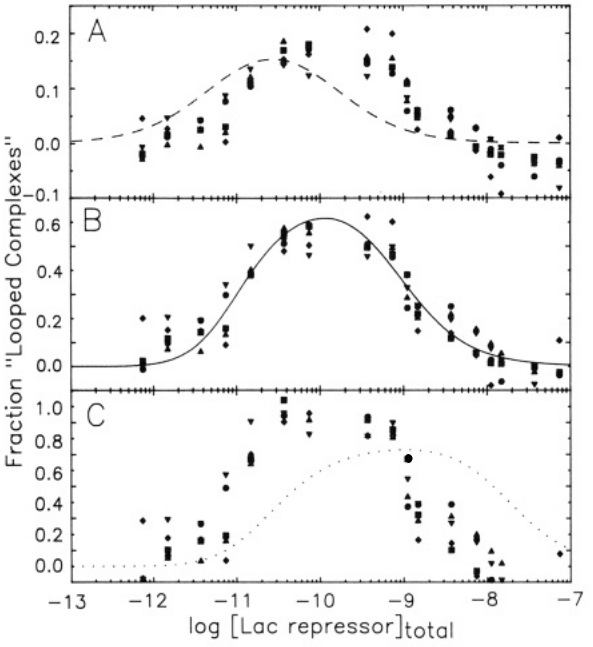

FIGURE 6: Fraction of looped complexes derived from quantitation of the DNase I hypersensitivity of bands located between the two binding sites in the O_E^L/O_I^L operator upon the binding of the Lac repressor. These data are derived from those shown in Figure 5 by numerical scaling of titration endpoints (eq 9) as described under Materials and Methods. The solid curves are derived from the best-fit values shown in Table IV from the simultaneous analysis of the titration data shown in Figure 3 with the DNase I hypersensitivity data shown in Figure 5. Panel A represents the results obtained assuming $\Delta G_{tet} = -25$ kcal/mol, panel B with −13.2 kcal/mol, and panel C with $\Delta G_{tet} = -10.6$ kcal/mol with the assumption that the DNA-binding affinities of dimers and tetramers are equal.

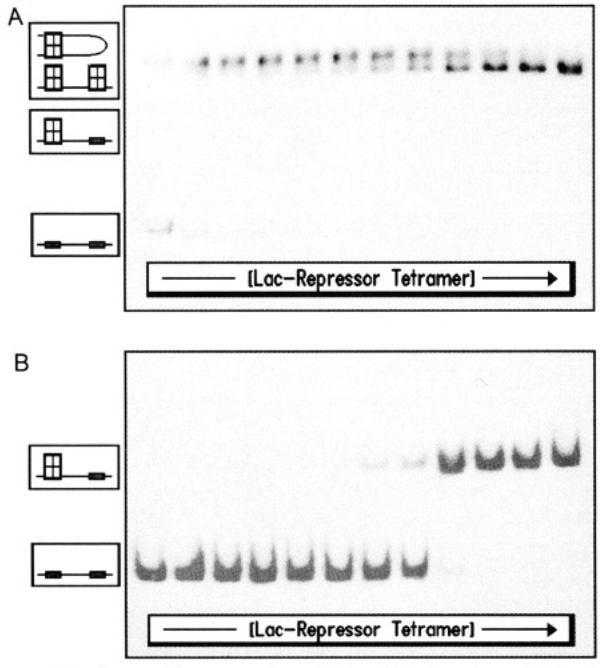

FIGURE 7: Autoradiograms of mobility-shift titrations of the (A) two-site O_E^L/O_I^L and (B) single-site O_E^L/O_I^G operators with the Lac repressor. Protein concentrations are, from left to right, (A) 1.4, 7.4, 14.8, 18.5, 37, 74, 148, 185, 370, and 740 pM and 1.5, 3.7, 37, 148, and 370 nM and (B) 0.0, 0.7, 1.5, 14.8, 18.5, 37, 74, 148, 370, and 740 pM and 1.5, 3.7, 14.8, 18.5, and 37 nM. The figure was produced from a digital representation of the autoradiograms by using techniques of image analysis.

DNA bending observed by Zwieb et al. (1989). In addition, separate dimer–DNA and tetramer–DNA complexes were not observed.

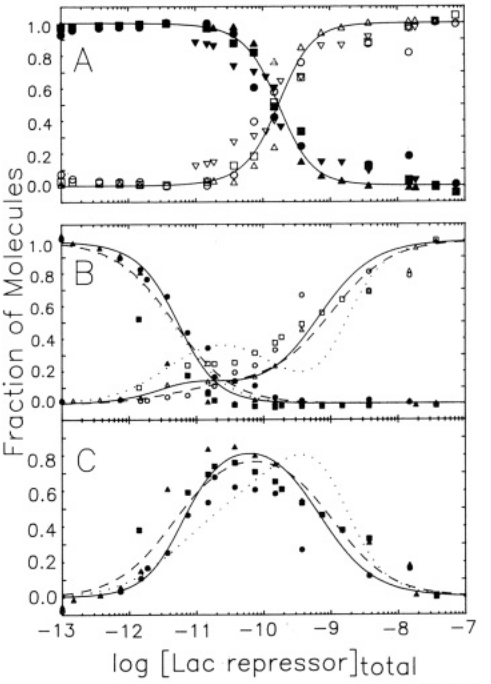

FIGURE 8: (A) Binding of the Lac repressor to the O_I^L/O_I^G single-site reduced-valency mutant operator. Solid symbols represent data derived from quantitation of the proportion of the unliganded DNA band, and open symbols represent the proportion of the band containing singly liganded operators. The solid lines indicate the binding curves calculated from the best-fit values shown in Table IV. Four separate titrations are shown. (B) Mobility-shift experiments of Lac repressor binding to the O_E^L/O_I^L operator. Solid symbols represent data for the unliganded DNA band, and open symbols represent the band containing tandem complexes; each symbol represents a separate experiment. (C) Binding curves representing the fraction of looped and singly liganded complexes (see text). Each symbol represents an individual experiment. Three separate experiments are depicted in B and C. The best-fit binding curves (Table IV) are represented by the dashed lines ($\Delta G_{tet} = -25$), solid lines ($\Delta G_{tet} = -13.2$), and dotted lines ($\Delta G_{tet} = -10.6$ kcal/mol).

In the titration of the two-site O_E^L/O_I^L operator, a transition from the "free DNA" band to two bands with retarded mobility occurred without the detectable presence of the singly liganded intermediate (compare Figure 7, panels A and B). Examination of the two bands whose mobility was retarded reveals that the initial appearance of the first band is followed by a transition, at higher protein concentrations, to a band of slightly increased mobility. As will be discussed below, the populations of the two bands are consistent with the transient appearance of looped complexes followed by the appearance of tandem complexes at higher protein concentrations (Kramer et al., 1987). That the electrophoretic mobilities of these complexes are so similar is consistent with the studies of Kramer et al. (1987), who demonstrated that the relative mobilities of the looped and tandem complexes vary as a function of the separation of the DNA-binding sites. When the binding sites are separated by 11 helical turns of 10.4 bp, as is true in the O_E^L/O_I^L operator, the mobility of the two complexes is almost identical.

Quantitation of autoradiograms of titrations of the O_E^L/O_E^G operator with the Lac repressor (Figure 7B) yielded titration curves for the free and singly liganded DNA (Figure 8A). The reproducibility of the experiment is shown by the agreement of the four experiments shown. Note that the free and complex bands both conform to the predicted binding curves over their entire range. A decrease in the apparent binding affinity was observed upon either very rapid or prolonged electrophoresis (data not shown); electrophoresis con-

Table V: Gel Mobility-Shift Analysis of the Binding of the Lac Repressor to the O_E^L/O_I^L and O_E^L/O_I^G Operators[a]

operator	ΔG_E	ΔG_j	ΔG_{tet}	s^b
O_E^L/O_I^G	−12.7 ± 0.2		(−25)	0.069
O_E^L/O_I^L	−13.2 ± 0.2	11.8 ± 0.2	(−25)	0.109
	−13.8 ± 0.2	12.1 ± 0.2	(−13.2)	0.113
	−14.3 ± 0.2	12.1 ± 0.4	(−10.6)	0.149

[a] The data depicted in Figure 8 were analyzed subject to the constraints that $\Delta G_E^d = \Delta G_E^t$ and $\Delta G_I^d = \Delta G_I^t$, i.e., that Lac repressor dimers and tetramers have equal DNA-binding affinity and that the DNA-binding affinity of the Lac repressor for O_E^L and O_I^L is identical. [b] Square root of the variance. Bracketed values do not differ significantly. [c] The dimer–tetramer association free energy of −25 represents nondissociable tetramer where the proportion of dimer is negligible over the entire titration range. Values in parentheses were fixed during the nonlinear least-squares analysis.

ditions were kept constant in order to minimize this potential artifact. The binding free energy determined from the analysis of the O_E^L/O_E^G titration with eqs 15–16 is approximately 1 kcal/mol less negative than the value determined from the corresponding footprint titrations (compare Table V, top line with Table IV, lines 1 and 3). That mobility-shift titrations report a weaker apparent binding affinity compared to footprint titrations has also been observed with the Gal repressor and a mutant Lac repressor (Brenowitz et al., 1990, 1991). However, the 1 kcal/mol discrepancy observed with the Lac repressor is twice that of the 0.5 kcal/mol difference observed with these other proteins. Footprint titrations of all three proteins were conducted in the presence and absence of the 3% glycerol present in the binding buffer for the mobility-shift experiments. That the pairs of titrations were identical suggests that the solution conditions do not account for the discrepancy between the two experimental techniques (data not shown). Because of this discrepancy, simultaneous analysis of the O_E^L/O_I^G and O_E^L/O_I^L gel mobility-shift titration data was not conducted.

Quantitation of the three bands observed upon titration of the two-site O_E^L/O_I^L is shown in Figure 8B,C. Since the separation of the two visible complex bands is slight, the boundary between the two bands was drawn at the minima of the density between them. That the faster mobility band of the two complex bands corresponds to the looped complex is clear from its transient appearance upon Lac repressor titration (Figure 8C). The slowest band is the tandem complex. This is clear from the its appearance only at high Lac repressor concentration, displacing the looped complex (Figure 8B). The density of the region in the O_E^L/O_I^L autoradiograms corresponding to the expected mobility of the singly liganded complex (Figure 7B) was also determined and included in the analysis. The titration data were fit to eqs 11, 12, 13, and 14 and analyzed under two constraints. The first constraint was that Lac repressor binds equivalently to O_E^L and O_I^L ($\Delta G_E = \Delta G_I \equiv \Delta G_{intrinsic}$). This constraint was justified based on the identity of the DNA-sequence of the two sites (Figure 1), the footprint titration results presented in Table IV (lines 1 and 3), and the fact that mobility-shift titrations of O_E^L/O_I^G (Table V, top line) and O_E^G/O_I^L (data not shown) are identical. The second constraint was that dimers and tetramers bind equivalently. Use of these constraints allowed the determination from the O_E^L/O_I^L titrations alone of $\Delta G_{intrinsic}$ and ΔG_j for assumed values of ΔG_{tet}. The results of this analysis revealed that for all assumed values of ΔG_{tet}, the fitted curves predicted measurable concentrations of the singly liganded intermediate (data not shown), in contrast to its absence on the autoradiograms (Figure 8A).

An explanation of this discrepancy is found in the transport simulations of Cann (1990) of the electrophoretic behavior of protein-mediated looped complexes. These simulations predict that the absence of a singly liganded band is due to the isomerization equilibria between the singly liganded and the looped complexes. This isomerization equilibrium was incorporated in the analysis of the O_E^L/O_I^L titrations by combining eqs 12 and 13 (eq 17, see Materials and Methods). The mobility-shift titration data for the O_E^L/O_I^L operator were fit to eqs 11, 14, and 17 by nonlinear least-squares analysis subject to the constraints described above. The data were unable to allow distinction between $\Delta G_{tet} = -25$ and −13.2 due to higher levels of error than were present in the footprint hypersensitivity data (Table V). Both sets of curves fit the data points reasonably well over their entire range (Figure 8, panels B and C, dashed and solid lines, respectively). However, the values of ΔG_E and ΔG_j resolved when ΔG_{tet} is assumed to be −13.2 kcal/mol are remarkably similar to the values obtained from the footprint titration and hypersensitivity curves (Table IV). In contrast, when $\Delta G_{tet} = -10.6$, the predicted curves systematically deviate from the data (Figure 8, panels B and C, dotted lines). However, note that ΔG_j is constant for all values of ΔG_{tet} (Table V).

Discussion

Protein-mediated DNA looped complexes are important regulatory elements in many systems. The stability of these complexes is the net result of several processes including the affinity of proteins for specific DNA sequences, protein self-association, increased local binding-site concentration, and DNA bending. An understanding of the biological role of DNA looping requires the quantitative characterization of each of these biochemical processes.

The individual-site loading free energies calculated from the footprint titrations represent the chemical work required to bind a protein to a DNA site (Ackers et al., 1983; Senear & Ackers, 1990). These loading energies include partial contributions from cooperative interactions involving that site. Although binding to the two-site operator is clearly cooperative, the −1 kcal/mol of cooperative interaction represents only about a 5-fold increase in the apparent binding affinity for linear DNA. This increase in apparent binding affinity is comparable with that observed by Whitson et al. (1986) in their comparison of the Lac repressor binding to linear DNA containing only the primary operator and linear DNA containing both the primary and pseudo-operators. If the bidentate Lac tetramer were the only species present in solution, this level of cooperativity would yield only a low concentration of looped complexes (Figure 3B, dashed line). The presence of a dimer–tetramer association constant whose magnitude is comparable to or less than the DNA-binding constants serves to increase the concentration of looped complexes that are present in solution *without* any increase in the apparent binding cooperativity. Thus, regulation of the dimer–tetramer association reaction could have potentially important regulatory consequences by decoupling looped complex formation from DNA binding. Support for this idea is found in a recent report suggesting that regulation of the *ara* operon is mediated by an alteration of the monomer–dimer association reaction of the AraC protein by arabinose (Lobell & Schleif, 1990).

It was not possible to determine unique values of both ΔG_j and ΔG_{tet} from the footprint titration studies in the absence of quantitation of the DNase I hypersensitive bands (Table III). In addition, determination of ΔG_j from mobility-shift titrations of O_E^L/O_I^L alone (i.e., without simultaneous analysis of O_E^L/O_I^L and O_E^L/O_I^G titrations) was dependent on the resolution of the bands representing the looped and tandem

complexes (Senear & Brenowitz, 1991). How accurate are these indicators of looped complexes? It is encouraging that the values of ΔG_j obtained from the two techniques are comparable. A limitation of the use of DNase I hypersensitivity as an indicator of looped complex concentration is the fact that the data yield only relative values (Figure 5); determination of the absolute magnitude is made by numerical scaling of the transition endpoints (eq 10). Thus, the information obtained from the DNase I hypersensitive bands resides in the position and the shape of the distribution at which the transition occurs (Figure 6). These facts were particularly useful in distinguishing different values of ΔG_{tet}. On the other hand, the mobility-shift assay directly yields the fraction of molecules that are in the form of looped complexes. However, an important concern with this assay is that, unlike the footprint titration experiment, this method depends upon the protein–DNA equilibria present in solution not being perturbed by the electrophoresis separation. This issue has been considered by several authors [cf. Carey (1987); Fried (1989); and Revzin (1989)]. Potential pitfalls in the quantitative analysis of bands representing liganded complexes have been presented by Cann (1989) on the basis of simulations of the electrophoretic transport of protein–DNA complexes coupled to reversible equilibria. Although we have not systematically explored this question with the Lac repressor/operator system, several observations suggest that the distribution of complexes is reported with reasonable accuracy in this analysis of mobility-shift experiments. The sum of the densities of the bands in each lane of the titration does not vary with protein concentration (data not shown). A decrease in the total DNA concentration would occur at partial values of fractional saturation due to dissociation of the complex resulting in "smearing" of the band density; none was observed in the autoradiograms. In addition, the bands representing both free and complexed DNA conform to the predicted binding curves over their *entire* range. Distortion of the shapes of the titration curves would be expected if the liganded complexes dissociated appreciably. However, the lower binding affinity for O_E^L/O_I^G reported by the mobility-shift titration experiment does suggest that caution must be observed in interpreting equilibrium constants obtained from these experiments. A more detailed analysis of the ability of gel mobility-shift experiments to resolve cooperativity and an evaluation of these issues will be presented elsewhere (Senear & Brenowitz, 1991).

The absence of a unique band corresponding to the singly liganded complex in the mobility-shift titrations of O_E^L/O_I^L is consistent with the transport simulations of Cann (1990). From the titration data representing the isomerization equilibria between the singly liganded and looped complex (Figure 8C), the fraction of looped complexes was calculated to reach approximately 65%, in excellent agreement with the footprint titration results (Figure 6B). The agreement of the free energies reported by the footprint titration experiments (Table IV) and the mobility-shift titration of O_E^L/O_I^L (Table V) support the validity of the transport simulations. A macroscopic "isomerization constant" was defined by Cann as the equilibrium constant of formation of looped complex from DNA containing a single tetramer, i.e., $K_{iso} = k_{intrinsic} \cdot k_j$. When the footprint and mobility-shift data were fit to binding expressions that were reparameterized by this definition of ΔG_{iso}, values of this constant of -1.4 ± 0.2 and -1.6 ± 0.2 kcal/mol were determined, respectively, when ΔG_{tet} is assumed to be -13.2 kcal/mol. These values correspond to isomerization equilibrium constants of 11.1 and 15.6 M^{-1}. It is clear from these values that the looped complex formed from this linear DNA fragment is only moderately more stable than the complex in which only a single protein–DNA contact is present.

Dimer–DNA and tetramer–DNA complexes could not be distinguished in these studies. The "retardation effect" of proteins on the electrophoretic mobility of DNA is a complex result of the net charge of the protein–DNA complex (Carey, 1987), the molecular weight of the protein [cf. Brown et al. (1990)], the length of the DNA, and the configuration of the complex [cf. Kramer et al. (1987); Zwieb et al. (1989)]. A comparison of the electrophoretic mobility of the Lac repressor and a number of mutant proteins that associate only to dimers revealed that the electrophoretic mobilities of their complexes with the 635-bp fragment employed in these studies are indistinguishable (J. Chen, D. Dalma-Weizhausz, K. S. Matthews, and M. Brenowitz, unpublished data). It should, of course, be considered that the dimer–tetramer association constant determined by Royer et al. (1990) predicts that the concentration of free tetramers is extremely small over the concentration range in which these studies were conducted. Additionally, if DNA-binding and dimer–tetramer association are linked, and tetramers bind DNA more tightly than dimers (Table IV, bottom line), then vanishingly small quantities of dimer–DNA complexes would be present in the equilibrium mixtures.

When the value of ΔG_{tet} of -10.6 kcal/mol determined by Royer et al. (1990) was assumed and ΔG_E^d, ΔG_I^d, ΔG_E^t, and ΔG_I^t were determined independently, the titration data converged to values where tetramers bound DNA with approximately 1.5 kcal/mol greater affinity than dimers (Table IV). This fit of the data was indistinguishable from the analysis conducted by assuming that $\Delta G^d = \Delta G^t$ with the determination of a value of ΔG_{tet} as a fitting parameter (Table IV). These results were unsurprising since the dimer–tetramer constant, ΔG_{tet}, and the intrinsic DNA-binding affinities of dimers and tetramers are highly correlated numerically. Are there additional data that allow us to distinguish these possibilities? The result that tetramers bind DNA with greater affinity than dimers (when $\Delta G_{tet} = -10.6$ kcal/mol) is inconsistent with the observations of Royer et al. (1990) that a Lac repressor–DNA complex is destabilized relative to the free protein. Studies of a Lac repressor mutant (I^{adi}) incapable of associating from dimers to tetramers show that it has an apparently unchanged DNA-binding affinity (Brenowitz et al., 1991), a result that suggests that dimer and tetramer affinity are similar, if not identical. On the other hand, cooperative binding of inducer in the presence of bound operator, but not in its absence, demonstrates DNA-linked heterotropic interactions within the Lac tetramer (O'Gorman et al., 1980). In addition, the loss of the wild-type pH dependence of inducer binding in a monomeric Lac repressor mutant suggests that this pH dependence is dependent on quaternary structure (Daly & Matthew, 1986). While we believe that the true value of ΔG_{tet} is closer to the value of -13.2 kcal/mol determined by assuming equivalent dimer and tetramer DNA-binding affinity, an independent determination of ΔG_{tet} by a different experimental technique and direct determination of linkage between DNA binding and dimer–tetramer association will be required to resolve this question.

The free energy term, ΔG_j, is a composite of several processes. These processes include bending DNA that is much shorter than the persistence length of the DNA (Hagerman, 1981; Rizzo & Schellman, 1981) and entropic terms relating to the local concentration of the second binding site. Despite the composite nature of ΔG_j, its determination does provide

the information necessary to determine the probability that a looped complex will form. The observation that ΔG_j for the *gal* DNA is 12.2 kcal/mol under these experimental conditions suggests an explanation for the absence of Gal repressor mediated looped complexes in the in vitro assays (Mandal et al., 1990; Brenowitz et al., 1990). The relative values of ΔG_E, ΔG_I, ΔG_j, and ΔG_{tet} determine the proportion of looped and tandem complexes present in solution. Values of ΔG_E and ΔG_I of -12.0 ± 0.2 kcal/mol were measured for Gal repressor binding to the O_E^G and O_I^G operators under these experimental conditions (Brenowitz et al., 1990). When the magnitude of $\Delta G_j \geq -\Delta G_{intrinsic}$, as is the case here, the predicted proportion of looped complexes is minimal. In addition, it is now clear that the Gal repressor has a limited, if any, propensity to self-associate into tetramers. Sedimentation equilibrium experiments with the Gal repressor revealed that tetramers were not detectable in Mg^{2+}-containing buffers at several conditions of temperature and pH (P. Hensley and M. Brenowitz, unpublished data). Thus, it is unlikely that any Gal repressor mediated looped complexes are present in solution under the conditions of the in vitro experiments. Taken together, these results suggest that it is unlikely that the Gal repressor by itself is capable of acting as a bidentate ligand and mediating the formation of a looped complex.

Since the Gal repressor appears unable to mediate formation of a looped complex, a question remains as to how the Lac repressor is able to substitute for the Gal repressor in the regulation of a modified *gal* operon (Figure 1; Haber & Adhya, 1988). Interpretation of this comparison of the Gal and Lac repressors rests on the assumption that overall geometry of the protein–DNA contacts of the two proteins are similar. Several lines of evidence suggest that this assumption is true. Both proteins specifically bind to DNA sites containing 2-fold symmetry as dimers using a "helix–turn–helix" motif [cf. Adhya (1989)], and both proteins alter the structure of the DNA by bending it upon binding (Wartell & Adhya, 1988; Zwieb et al., 1989). Thus, the primary differences between the Gal and Lac repressors as "DNA-looping proteins" appear to be their DNA-binding affinities and dimer–tetramer association constants.

A structural explanation for this conclusion is found in a comparison of the amino acid sequences of the Gal and Lac repressors, which shows that the Gal repressor is lacking the 15 C-terminal residues of the Lac repressor (von Wilcken-Bergmann & Muller-Hill, 1982). Deletion of this region from the Lac repressor results in a loss in the ability of the protein to form tetramers and mediate formation of looped complexes (Oehler et al., 1990; Mandal et al., 1990; Brenowitz et al., 1991). That the three-leucine heptad repeat located within this region acts as a "leucine zipper motif" (Landschulz et al., 1988) mediating the dimer–tetramer interaction was recently demonstrated by Chakerian et al. (1991) and Alberti et al. (1991). Thus, it appears likely that an additional component or components must be involved if the Gal repressor participates in the formation of a looped complex.

The thermodynamics of DNA bending have been defined by measurement of the solution properties (Hagerman, 1981; Rizzo & Schellman, 1981) and cyclization probabilities (Shore & Baldwin, 1983; Shimada & Yamakawa, 1984; Taylor & Hagerman, 1990) of DNA restriction fragments. However, protein-mediated DNA loops differ from circular DNA in several ways. First, for small protein-mediated DNA loops, the protein is of comparable size to the DNA loop. The length of a Lac repressor tetramer is approximately 125–130 Å (Steitz, 1990). Thus, the actual degree of the DNA bend is

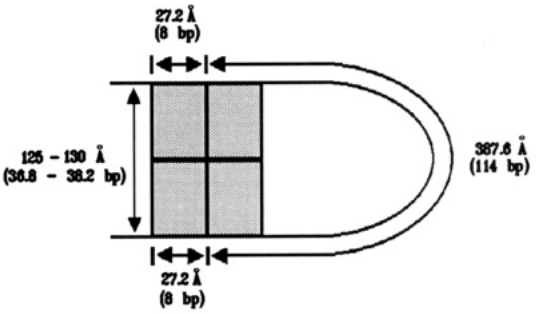

FIGURE 9: Schematic representation of a Lac repressor mediated looped complex. Approximate distances are given in angstroms. Base-pair values and distances were related assuming a value of 3.4 Å per base pair of axial rise. This model assumes that the Lac repressor subunits have a planar geometry (Steitz, 1990) and neglects any details concerning the geometry of the protein–DNA interaction.

significantly reduced compared to the cyclization of a free DNA fragment. Second, the molecular details of the protein–DNA interaction might create geometric constraints. Protein-induced DNA bends and the angle of passage of the DNA helix across the protein, the "loop-crossover angle", might significantly alter the energetics of the loop [cf. Zwieb et al. (1989); and Snyder et al. (1989)]. The effect of the "loop-crossover angle" on cyclization probabilities has been quantitatively considered by Hagerman and Ramadevi (1990). Third, the presence of DNase I hypersensitivity in the DNA between the protein-binding sites indicates that the structure of the DNA is altered within the looped complex. The deformation of the DNA from a B conformation might be an important component in the stability of small DNA loops [cf. Borowiec et al. (1987)].

How does the cyclization of free DNA and protein-mediated looped complexes compare? Neglecting torsional effects [cf. Hagerman and Ramadevi (1990)], values of the cyclization probability j of the DNA of 5.4×10^{-11}, 0.8×10^{-9}, and 7.5×10^{-9} were calculated from ΔG_j when ΔG_{tet} was assumed to be -25, -13.2, and -10.6 kcal/mol, respectively (Table IV). The small value of j determined when $\Delta G_{tet} = -25$ kcal/mol precludes an accurate estimation of number of DNA base pairs to which it corresponds by extrapolation from the theoretical curves describing the data of Shore and Baldwin (1983) (Hagerman, 1985; Hagerman & Ramadevi, 1990). The values of j determined when ΔG_{tet} was assumed to be -13.2 and -10.6 kcal/mol correspond to approximately 16 helical turns (166 bp) and 22 helical turns (228 bp), respectively, of DNA (Shimada & Yamakawa, 1984; Hagerman, 1985), lengths significantly longer than the 11 helical turns (114 bp) between the two protein-binding sites (Figure 1). Indeed, the cyclization probability of 114 bp of DNA is vanishingly small (Shimada & Yamakawa, 1984; Hagerman, 1985).

In order to compare the values of j determined for free DNA with the values determined for the Lac repressor mediated looped complex, consider the model diagrammed in Figure 9. In this simple model, the 130-Å distance separating the two Lac repressor DNA-binding sites is represented by an additional segment of DNA, resulting in an "apparent circumference" of the complex of approximately 16.1 helical turns (167 bp). Given the simplistic assumptions made in the model, the agreement with the value of 16 helical turns calculated when $\Delta G_{tet} = -13.2$ is remarkable. This result suggests that the DNA within the protein–DNA looped complex has similar thermodynamic properties when compared to free DNA. However, further analysis of values of ΔG_j for other protein-mediated looped complexes as well as structural studies to determine the geometry of the protein–DNA interaction

will be required in order to accurately relate the structure and energetics of looped complexes.

How do these results compare with the thermodynamic properties of looped complexes in vivo? Additional factors that can affect the formation and stability of looped complexes include DNA sequence elements (Goodman & Nash, 1989), the binding of DNA-bending proteins (Goodman & Nash, 1989; Moitoso de Vargas et al., 1989), DNA supercoiling (see below), and the spacing and orientation of the protein-binding sites on the DNA (Bellomy et al., 1988). In vivo characterization of aspects of the thermodynamics of Lac repressor mediated DNA looping have been conducted by Record and co-workers (Bellomy et al., 1988). Their observation of a decrease in the apparent persistence length and a change in the helical repeat of the DNA, in vivo, suggests that these additional factors have important regulatory consequences. The suggestion that the decrease in the apparent persistence length is due to the binding of catabolite activator protein (CAP) between the primary and secondary operators of the *lac* operon (Bellomy et al., 1988) is supported by the recent report of cooperative binding of CAP and the Lac repressor (Hudson & Fried, 1990). However, this latter study did not directly demonstrate stabilization of the Lac repressor mediated looped complex by CAP. In contrast to these results, simultaneous binding studies of the Lac repressor and CAP to the DNA shown in Figure 1 have failed to demonstrate stabilization of the looped complex by CAP (Dalma-Weiszhausz et al., 1991). The effect of CAP at the *gal* operon will result from the relative spacing and phase of the protein-binding sites as well as the DNA bending induced by CAP.

Studies conducted on several systems, including the *lac* operon (Borowiec et al., 1987; Whitson et al., 1987a,b; Kramer et al., 1988; Eismann & Muller-Hill, 1990; Bellomy et al., 1988) and the *ara* operon (Hahn et al., 1986; Lobell & Schleif, 1990), show that supercoiling increases the stability of the looped complexes as well as alters the helical repeat and apparent persistence length of the DNA [see Bellomy et al. (1988) for a discussion of these issues]. It is interesting that supercoiling does not appear to affect the torsional rigidity of the DNA (Bellomy et al., 1988). Thus, to a great extent, the requirement that the protein-binding sites be on the same face of the DNA helix, i.e., "phased" correctly, is preserved in supercoiled DNA. It is clear from an examination of the energies that define the stability of a Lac repressor mediated looped complex (e.g., Table I, species 8) that any process that increases the cyclization probability (decreases ΔG_j) would greatly increase the stability of the looped complex. In fact, in the limit of "infinite cyclization probability" ($\Delta G_j = 0$), the free energy of the tetramer-stabilized looped complex, ΔG_{s8} (Table I), would be $\Delta G_E + \Delta G_I$ or −27.5 kcal/mol. A complex of this stability ($k_d = 3 \times 10^{-21}$) would be essentially nondissociable. Thus, if supercoiling increases the cyclization probability of DNA, as seems likely based on the studies referenced above, then a combination of a high degree of negative supercoiling and protein-mediated DNA looping could serve to essentially inactivate genes.

Despite the ubiquitous occurrence of looped complexes, the mechanism(s) by which they inhibit transcription initiation when none of the protein-binding sites overlap the promoter region remain(s) unknown. The ability to accurately assess the roles of protein self-association and DNA cyclization probability in determining the energetic stability of looped complexes will allow the detailed dissection of the structure–function and thermodynamic relationships that underlie their formation and stability. Further study of this model "looping" system will ultimately allow quantitative correlations to be drawn between the thermodynamics of the formation of looped complexes and the ability of such a complex to regulate transcription initiation.

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