Modulation of the Stability of a Lac Repressor Mediated Looped Complex by Temperature and Ions: Allosteric Regulation by Chloride†

Michael Brenowitz* and Elizabeth Jamison

Department of Biochemistry, The Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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ABSTRACT: The lactose repressor of *Escherichia coli* (LacI) associates to a bidentate tetramer in solution and can simultaneously bind two operators to form a protein-mediated "looped complex". Studies have been conducted of the binding of LacI to two operators separated by approximately 11 helical turns of DNA. Quantitative DNase I footprint titration analysis of the stability of the LacI-mediated looped complex reveals that the Gibbs free energy of cyclization ($\Delta G°_j$) of the looped complex of 11.7 ± 0.4 kcal/mol is invariant with temperature. van't Hoff analysis reveals a large and positive enthalpy of cyclization ($\Delta H°$ = 12.3 ± 2.4 kcal/mol) and an entropy that is small and positive ($\Delta S°$ = 2.2 cal/deg). Quantitative DNase I footprint titration and kinetic dissociation studies were also conducted as a function of counter-ion type and concentration. Increasing concentrations of KCl or potassium glutamate destabilize the looped complex, a result completely accounted for by increases in the intrinsic DNA-binding free energies. While the value of $\Delta G°_j$ is invariant with ion concentration, chloride is a positive regulator. The value of $\Delta G°_j$ decreases by 1.5 kcal/mol upon substitution of chloride for glutamate. Measurements of $\Delta G°_j$ conducted as a function of chloride concentration at constant ionic strength reveal that approximately one chloride ion per tetramer is bound upon looped complex formation. These results demonstrate specific allosteric regulation of the formation of the LacI-mediated looped complex by a mechanism distinct from the regulation of the constituent protein–DNA interactions.

A ubiquitous mechanism by which the initiation of gene transcription is regulated involves the binding of a bidentate protein to two widely spaced sites on the DNA to form a protein–DNA "looped complex". The lactose repressor of *Echerichia coli* (LacI)[1] is a well-characterized model for such looped complex formation [cf. Adhya (1989), Bellomy and Record (1990), Matthews (1992), and Schleif (1992)]. LacI is a member of an extensive family of bacterial repressors that bind specific DNA sequences as dimers utilizing the "helix–turn–helix" motif (von Wilcken-Bergmann & Müller-Hill, 1982; Weickert & Adhya, 1992), although LacI is the only member of this family that has been shown to associate to tetramers in solution and to simultaneously bind two DNA recognition sequences, on either the same molecule or different molecules. The stability of the looped complex that is formed when LacI binds to two sequences on a contiguous piece of DNA is dependent on several classes of interactions. These interactions include the protein–DNA interaction of each dimer within the tetramer, the self-association equilibria of the binding protein, and the flexibility of the intervening DNA.

In order to probe the contribution of each of these interactions to the stability of a LacI-mediated looped complex, a series of quantitative DNase I footprint titration and kinetic dissociation studies have been conducted as a function of temperature and counter-ion type and concentration. These studies demonstrate that (1) the free energy of cyclization of the looped complex is constant with temperature, and the stability of the complex tracks with the changes in binding free energy of LacI for the DNA; and (2) while the decrease in the stability of the LacI-mediated looped complex with increasing ionic strength can be completely accounted for by more positive *intrinsic* DNA-binding free energies, the type of counter-anion present in solution has a significant effect on the stability of the complex. Specifically, the free energy of cyclization ($\Delta G°_j$) of the LacI-mediated looped complex is more negative when chloride is substituted for glutamate as the counter-anion. This decrease occurs despite increases in the affinity of LacI for DNA in glutamate-containing solutions (Leirmo et al., 1987; Ha et al., 1992). Chloride is a positive allosteric regulator of LacI-mediated looped complex formation. The implications of these results for the regulation of cellular processes by looped complexes will be discussed.

MATERIALS AND METHODS

Preparation of Protein and DNA. The preparation of linear DNA restriction fragments of 635 bp containing the sequences from −199 to 434 relative to the S1 transcription start site of the *gal* operon has been described (Brenowitz et al., 1990, 1991a). These DNA molecules contain chimeric *gal* control regions in which the Gal repressor recognition sequences (designated O_E^G and O_I^G) are altered to LacI recognition sequences (designated O_E^L and O_I^L) (Haber & Adhya, 1988). The LacI recognition sequence is TTGTGAGC·GCTCACAA, which is an inverted repeat of 8 base pairs of the left half of the wild-type operator (Sadler et al., 1983). The two operators are separated by 113 bp, or 10.9 helical turns (10.4 bp/turn). Studies were conducted with DNA in which both operators were recognized by LacI (O_E^L/O_I^L) or with DNA in which only one site was recognized by LacI (O_E^G/O_I^L or O_E^L/O_I^G).

LacI was a gift of Dr. Kathleen Matthews and was prepared as described in Rosenberg et al. (1977) and O'Gorman et al. (1980). Lac^{adi} was prepared as described in Brenowitz et al. (1991b). The specific DNA-binding activity of the LacI is

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* Author to whom correspondence should be addressed. Telephone (718) 430-3179. Email: brenowit@murex.bioc.aecom.yu.edu

[1] Abbreviations: *E. coli* LacI, *Escherichia coli* Lac repressor; glu, glutamate; DTT, dithiothreitol; BSA, bovine serum albumin; bp, base pair.

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92 ± 12%. All reagents used are reagent or molecular biological grade.

DNase I Footprint Titration Analysis. Quantitative DNase I footprint titration experiments were conducted as described in Brenowitz et al. (1991a) and the references cited therein. A buffer (footprint buffer A) containing 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 7.00 with HCl at the indicated temperature was used in the temperature dependence studies. Experiments in which the KCl concentration was varied were conducted at 20 °C and utilized the same buffer composition described above except that the KCl concentration was as indicated. Experiments employing glutamate as the counter-anion were conducted in 25 mM Bis-tris, 5 mM magnesium acetate, 1 mM calcium acetate, 2 mM DTT, 50 μg/mL BSA, 2 μg/mL calf thymus DNA, and the indicated concentrations of KGlu titrated to pH 7.00 with glutamic acid at 20 °C (footprint buffer B). Footprint buffer B was used in studies conducted as a function of Cl^- concentration except that KCl was present at the indicated concentrations and KGlu was added to maintain the monovalent ion concentration at 100 mM. In all the footprint titration experiments, the protein and DNA were equilibrated for 30–45 min prior to the addition of DNase I. Presiliconized microfuge tubes (Low-Binding Lube Tubes from Marsh Biomedical Products) were used at all steps in the experimental protocols. The assumption that $[LacI]_{total} \approx [LacI]_{free}$ was made in all experiments since the concentration of operator sites in the equilibrium mixtures was low relative to the protein–DNA dissociation constants.

The formulation of a statistical mechanical model describing the interaction of the bidentate LacI tetramer to two binding sites widely separated on the DNA and of the individual-site binding equations appropriate for these titration experiments has been described (Brenowitz et al., 1991a). In this model, the binding of LacI is described by four free energies. Two *intrinsic* binding free energies, $\Delta G°_E$ and $\Delta G°_I$, describe the binding of LacI to O_E^L and O_I^L, respectively, in the absence of other interactions. $\Delta G°_j$ represents the "free energy of *cyclization*" of the looped complex and is defined by analogy to the "cyclization probability" (j factor) [cf. Shore and Baldwin (1983a,b) and Shimada and Yamakawa (1984)] of the DNA within the protein–DNA complex by the expression $\Delta G°_j = -RT \ln j$, where R is the gas constant and T is the temperature in degrees Kelvin. $\Delta G°_j$ is defined as the energy difference between the looped complex (in which a single LacI tetramer bridges the two binding sites) and the "tandem complex" (in which a LacI tetramer is bound to both DNA sites). An alternative way to describe the stability of the looped complex is by an *isomerization* free energy, $\Delta G°_{iso}$, that represents the energy difference between the looped complex and the complexes that have a LacI tetramer bound to a single site. These two free energies are related by the expression $\Delta G°_{iso} = \Delta G°_{intrinsic} + \Delta G°_j$ (Cann, 1990).

The fourth energy needed to describe the system in this model is the dimer–tetramer association free energy, $\Delta G°_{tet}$. The concentrations of LacI dimers and tetramers were calculated by assuming that 2 Dimer ⇄ Tetramer is the sole equilibrium necessary to describe the self-association of the protein in the concentration range used (Royer et al., 1990). The value of $\Delta G°_{tet}$ of −13.2 kcal/mol determined from the analysis of DNase I footprint titrations and looped complex-induced DNase I hypersensitivity (Brenowitz et al., 1991a) was used in the calculations unless otherwise indicated.[2] The determination of this value of $\Delta G°_{tet}$ (in KCl-containing buffer) was subjected to the assumption that LacI dimers and tetramers bind DNA equivalently and that the LacI tetramer binds DNA non-cooperatively (see Discussion).

The resolution of the two intrinsic free energies and the cyclization (or isomerization) free energy requires the simultaneous analysis of the footprint titrations determined for LacI binding to the DNA containing two binding sites (O_E^L/O_I^L) and of the DNA separately containing each individual site (O_E^G/O_I^L and O_E^L/O_I^G). However, since O_I^L and O_E^L are identical in sequence and have almost identical affinity for LacI (Brenowitz et al., 1991a), the assumption that $\Delta G°_E = \Delta G°_I \equiv \Delta G°_{intrinsic}$ was made and simultaneous analysis of titrations of O_E^L/O_I^L and O_E^L/O_I^G were conducted to resolve values of $\Delta G°_{intrinsic}$ and $\Delta G°_j$ (or $\Delta G°_{iso}$). Common values of the free energies are obtained which describe the least-squares minima for the analysis of all three titration curves (Ackers et al., 1982; Senear & Ackers, 1990).

Equilibrium Binding by the Nitrocellulose Filter-Binding Assay. Nitrocellulose filter binding (Riggs et al., 1970) was used to determine the affinity of LacI for a single specific site (O_E^L/O_I^G). Titrations were conducted in footprint buffer A without the calf thymus DNA (filter-binding buffer A). S & S 40-μm nitrocellulose filters (HAWP 02500) were prewetted with filter-binding buffer A without BSA (wash buffer) for several hours. Samples of 100 μL containing the [^{32}P]DNA and LacI were equilibrated in filter-binding buffer A at 20 °C for 45 min. Aliquots of 95 μL were drawn through a filter at a rate of 1.0 mL/min at room temperature. Constant flow rates were obtained by use of a peristaltic pump. The filters were washed once with 500 μl of wash buffer and placed in a scintillation vial. The filters were soaked in 5 mL of scintillation fluid overnight prior to counting. The filter-binding titrations of LacI with the specific site were analyzed as has been described (Senear et al., 1986).

Dissociation Kinetics by the Nitrocellulose Filter-Binding Assay. The dissociation of LacI–DNA complexes was monitored using nitrocellulose filter binding to detect the fraction of protein–DNA complexes remaining as a function of time. LacI was equilibrated with ~10 fM [^{32}P]DNA in 100 μL of a solution containing either footprinting buffer A or B without the calf thymus DNA. The samples used in the dissociation experiments were equilibrated at 20 ± 0.1 °C for ~30 min. At time 0, unlabeled plasmid DNA containing two high-affinity sites for LacI (plasmid pH107; Haber & Adhya, 1988) was added to ~2 nM concentration. At the indicated times 95-μL aliquots were drawn through a filter as described in the preceding section. The fraction of counts retained on the filter at each time is expressed relative to the counts retained at time 0. The dissociation progress curves were fit to either a single exponential,

$$f(t) = e^{-k_1 t} \quad (1)$$

[2] It must be emphasized that this value of the LacI dimer–tetramer association free energy is an estimate determined from DNA-binding studies and is subject to several assumptions. Although not stressed in the original publication (Brenowitz et al., 1991a), the value of −13.2 kcal/mol is best regarded as an upper limit to the value of ΔG_{tet}. Recently, Fickert and Müller-Hill (1992) have presented a different indirect assay, differential retention of different LacI–DNA complexes on nitrocellulose filters, and claim a K_d of 3–5 pM for the dimer–tetramer association. However, it should be noted that this assay is also dependent upon several assumptions regarding the binding of DNA by LacI and was conducted at a very low salt concentration. The varied values observed for the dimer–tetramer association constant by different techniques highlight the necessity of directly obtaining thermodynamically rigorous values for the association reaction under a range of experimental conditions in order to resolve the ambiguity in this issue.

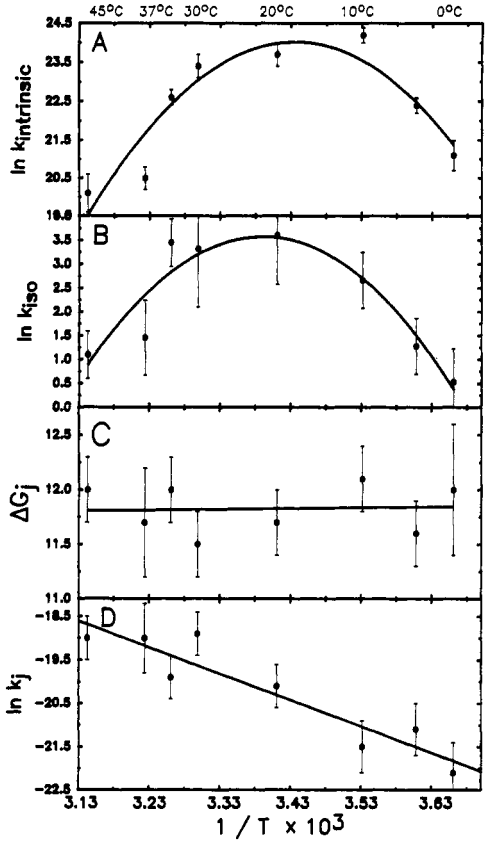

FIGURE 1: van't Hoff plots of (A) the intrinsic DNA-binding constant ($k_{intrinsic}$), (B) the isomerization constant of the looped complex (k_{iso}), and (D) the cyclization probability (k_j) determined from the quantitative footprint titration studies described in Materials and Methods. (C) A plot of the cyclization free energy, $\Delta G°_j$, vs $1/T$.

or the sum of two exponentials,

$$f(t) = Ae^{-k_2 t} + (1 - A)e^{-k_1 t} \quad (2)$$

where k_1 and k_2 are the first-order dissociation constants, t is time in seconds, and A is the fraction of the second component, using techniques of nonlinear least-squares analysis (Johnson & Frasier, 1985). The confidence limits reported correspond to approximately 1 SD.

RESULTS

Temperature Dependence of the Cyclization of the LacI-Mediated Looped Complex. A series of DNase I footprint titrations were conducted of LacI binding to DNA containing two specific sites separated by 11 helical turns (O_E^L/O_I^L) and to DNA containing only one specific site (O_E^L/O_I^G) in order to resolve the DNA-binding and -cyclization (or isomerization) free energies. The data were analyzed using the model for the association of a bidentate ligand binding to two specific sites described in Brenowitz et al. (1991a). The dimer–tetramer association free energy, $\Delta G°_{tet}$, has been shown to be invariant over at least a part of the temperature range encompassed by these studies (Royer et al., 1990) and is assumed to be constant above 20 °C (see below). The nonlinear van't Hoff plot that is observed for the *intrinsic* binding free energy ($\Delta G°_{intrinsic}$) of the LacI–operator interaction (Figure 1A) is comparable to that determined from nitrocellulose filter-binding studies of LacI binding to a single specific site by Record and co-workers (Ha et al., 1989). Ha et al. interpreted their data to represent a large negative standard heat capacity change, ΔC_p, in the association of LacI with DNA and proposed that the negative ΔC_p results primarily from the removal of nonpolar surface with water. The *isomerization* ($\Delta G°_{iso}$) of the LacI-mediated looped complex shows a comparable nonlinear dependence upon temperature and tracks very closely with the changes in *intrinsic* affinity of the protein for the operator (Figure 1B). The reason for this correspondence is seen when the data are analyzed in terms of the free energy of cyclization ($\Delta G°_j$) rather than isomerization ($\Delta G_{iso} = \Delta G°_{intrinsic} + \Delta G°_j$; see *Materials and Methods*); the values of $\Delta G°_j$ are invariant with temperature (Figure 1C). The van't Hoff plot of the cyclization constant, k_j ($\Delta G°_j = -RT \ln k_j$), is linear, yielding a value of $\Delta H°$ of 12.3 ± 2.4 kcal/mol (Figure 1D). An enthalpy per base pair of 0.11 kcal/mol is calculated if it is assumed that the DNA loop is bent smoothly along the entire 113 bp constituting the center to center distance between O_E^L and O_I^L.

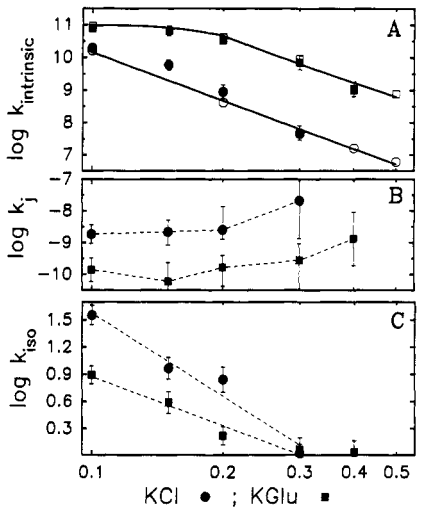

FIGURE 2: Dependence of (A) the intrinsic binding constant ($\Delta G°_{intrinsic}$), (B) the cyclization probability (k_j), and (C) the isomerization constant (k_{iso}) on KCl (●) and KGlu (■) concentration. The open symbols in panel A denote results from nitrocellulose filter-binding experiments. The solid symbols denote the results resolved from the quantitative footprint titration studies.

Ion Dependence of the Cyclization of the LacI-Mediated Looped Complex. The dependence of LacI binding to a single site on the type and concentration of monovalent ions has also been extensively characterized by Record and co-workers [cf. Ha et al. (1992) and references cited therein]. A log–log plot of the results of nitrocellulose filter-binding analysis of LacI binding to the single-site O_E^L/O_I^G DNA shows a linear dependence between 100 and 500 mM KCl and between 200 and 500 mM KGlu (Figure 2A, open circles). (These titrations were conducted at sufficiently high monovalent ion concentrations to render negligible to the monovalent ion dependence the contribution of the 5 mM Mg^{2+} and 1 mM Ca^{2+} present in the binding buffer that is required for DNase I activity.) In the absence of divalent cations, the value of $-d \log k/d \log [MX]$ is equal to the net number of ions released upon the formation of the protein–DNA complex. In the presence of divalent ions, the number of ions released is approached by the limiting slope at high monovalent ion concentrations. A value of 4.9 ± 0.2 was determined for the binding of LacI to O_E^L under these experimental conditions.

The *intrinsic* binding free energies resolved from the simultaneous analysis of DNase I footprint titration studies of LacI binding to the single-site, O_E^L/O_I^G and the two-site, O_E^L/O_I^L DNA molecule are identical, within experimental error, with the values determined by filter binding (Figure 2A, solid circles). The values of k_{iso} decrease with increasing ion concentration in both KCl- and KGlu-containing buffers.

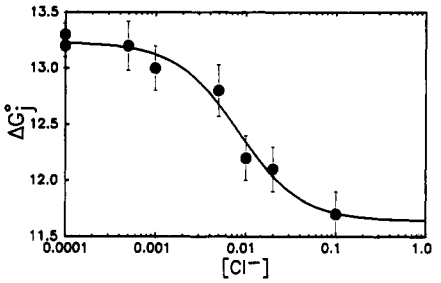

FIGURE 3: Dependence of the cyclization free energy (ΔG°_j) on the concentration of chloride ions when the total monovalent ion concentration is maintained at 100 mM by the addition of KGlu. Values determined from quantitative footprint titration studies as described in the text.

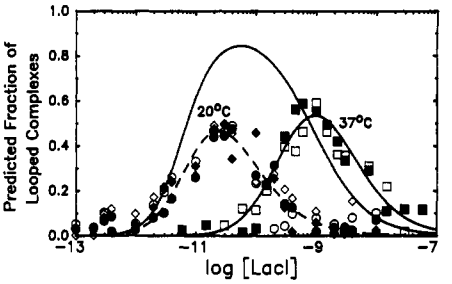

FIGURE 4: Predicted fraction of LacI-mediated looped complexes present in solutions containing 100 mM KCl at 20 and 37 °C (solid lines) and 100 mM KGlu at 20 °C (dashed line). The symbols represent quantitation of individual bands present in the intervening DNA that become hypersensitive to DNase I cleavage upon the formation of a looped complex. These data were analyzed as described in Brenowitz et al. (1991a). The DNase I hypersensitivity data obtained at 20 °C in KCl-containing buffer have been published previously (Brenowitz et al., 1991a) and are omitted for clarity.

That the values of k_{iso} track with $k_{intrinsic}$ is clearly seen by the linearity of the log–log plot from 0.1 to 0.3 M KCl (Figure 2B, solid circles). As seen in the temperature-dependence studies, the dependence of k_{iso} on $k_{intrinsic}$ is reflected in the fact that ΔG°_j is unaffected by the monovalent ion concentration (Figure 2C). (It should be noted that this assay is insensitive to the value of ΔG°_j when its magnitude is equal to or exceeds $-\Delta G^\circ_{intrinsic}$. This fact is reflected in the broadening confidence limits at 0.3 M KCl (Figure 2C, solid circles).)

Despite the fact that the DNA-binding affinity of LacI is greatly *increased* when Glu^- rather than Cl^- is the counter-anion (Figure 2A), the stability of the looped complex is significantly *decreased* in Glu^--containing buffer (Figure 2B). For example, in 100 mM KCl, k_{iso} is 37.1 ± 1.4 ($\Delta G^\circ_{iso} = -2.1$ ± 0.2), and in 100 mM KGlu, k_{iso} is 7.9 ± 2.0 ($\Delta G^\circ_{iso} = -1.2$ ± 0.4). This unexpected destabilization is dramatic; a value of $k_{iso} \simeq 145$ ($\Delta G^\circ_{iso} = -2.9$) was predicted for LacI-mediated looped complex formation in 100 mM KGlu relative to KCl, on the basis of the increased DNA-binding affinity of LacI in KGlu. Analysis of the titration data for the apparent cyclization free energy, ΔG°_j, reveals that its value is essential constant as a function of salt concentration. However, its value in KGlu is substantially more positive than in KCl. Since it is unlikely that the exchange of Glu^- for Cl^- affects the intrinsic flexibility of the DNA (see Discussion), it is clear that an additional process must be contributing to the increase in ΔG°_j.

Linkage of Chloride Ion Binding with ΔG°_j. A series of footprint titrations were conducted in which the chloride ion concentration was varied and the monovalent ion concentration was held constant at 100 mM by the addition of KGlu. The values of ΔG°_j determined as a function of [Cl^-] are shown in Figure 3. The data clearly show that the binding of chloride ions accompanies the formation of a LacI-mediated looped complex. The chloride-linked contribution to ΔG°_j is equal to $\Delta G_j^{[Cl^-]} - \Delta G_j^{ref}$, where the superscripts indicate the ion concentration of interest, and the reference state (ref) is a concentration at which ΔG°_j is independent of [Cl^-], in this case below 0.1 mM. A value of 1.5 kcal/mol is calculated from these data. The slope of the transition shown in Figure 3 is related to the net absorption of chloride ions by $\partial(\ln k_j)/\partial(\ln a_{Cl}) = \Delta\overline{Cl}$ (Wyman, 1964), where k_j is the cyclization probability, a_{Cl} is the chloride activity, and $\Delta\overline{Cl}$ is the change in the amount of ligand bound that accompanies the reaction. A value of $\Delta\overline{Cl}$ of 0.8 ± 0.3 is calculated from the data. These data are consistent with the uptake of one chloride ion per tetramer upon formation of a looped complex.

That the observed modulation of ΔG°_j is specific to chloride ions was demonstrated in two ways. First, a value of ΔG°_j of −13.4 ± 0.6 was determined in buffer containing 100 mM potassium acetate. Acetate appears to compete with chloride since a value of ΔG°_j of −13.3 ± 0.5 was determined in buffer containing 15 mM KCl and 85 mM potassium acetate. Second, decreasing the concentration of KGlu from 100 to 50 mM did not result in any change in ΔG°_j.

Quantitation of Looped Complex Formation. The fractions of looped complexes calculated to be present in solution at 20 and 37 °C in KCl (solid lines) and at 20 °C in KGlu (dashed line) are shown in Figure 4. The decrease in the fraction of looped complexes at high concentrations of LacI is due to mass-action-driven formation of tandem complexes, in which a LacI tetramer is bound to each of the two operators (Krämer et al., 1987). Densitometric analysis of the bands in the intervening DNA that become hypersensitive to DNase I upon looped complex formation have been used to directly determine the concentration dependence of looped complex formation (Brenowitz et al., 1991a). The concentration distribution was then used to estimate a value of ΔG°_{tet} at 20 °C (Brenowitz et al., 1991a; see Discussion). As the dimer–tetramer association interaction weakens, the concentration at which looped complexes form increases relative to the individual-site binding isotherms. Although direct self-association studies are required to resolve this issue, the fact that the DNase I hypersensitivity data at 37 °C are consistent with the predicted concentration distribution of looped complexes (Figure 4, solid line and squares) suggests that ΔG°_{tet} does not change appreciably at elevated temperatures. This result is consistent with the high-pressure dissociation studies conducted at lower temperatures (Royer et al., 1990). A possible explanation of the increase in the value of ΔG°_j in KGlu- relative to KCl-containing buffers is that the counter-anion affects the dimer–tetramer association equilibrium. However, the analysis of the DNase I hypersensitivity data for the titrations conducted at 100 mM KGlu suggests that ΔG°_{tet} is also not significantly affected by the substitution of KGlu for KCl (Figure 4, dashed line).

Dissociation Kinetics of LacI from a Single Specific Site. In order to provide an independent determination of the stability of the looped complex, the rates of dissociation of LacI from DNA containing either one or two operators were determined. The concentration of operator DNA present in the reaction mixtures is sufficiently low to preclude the formation of "sandwich complexes", in which a single LacI tetramer binds two molecules of DNA. Progress curves were first obtained for the dissociation of LacI from DNA containing a single specific site over a range of initial concentrations of

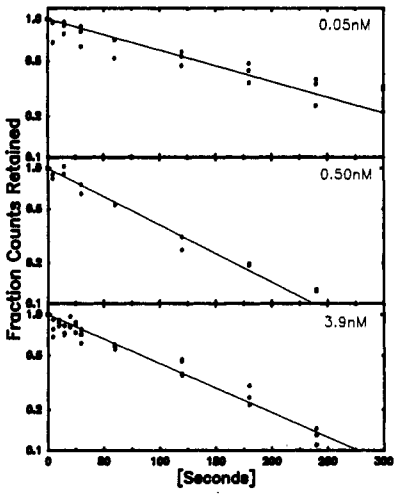

FIGURE 5: Dissociation progress curves for LacI bound to a single specific DNA site as a function of initial protein concentration. Data was collected until the fraction of the counts retained on the filter reached 10%.

LacI (Figure 5). At protein concentrations less than or equal to the equilibrium dissociation constant, the dissociation progress curves are described by a single exponential (Figure 5, 0.05–3.9 nM). Consistent values of k_1 are resolved over an ~80-fold range of initial LacI concentrations (Table I, lines 1–3). An average value of k_1 of 7.7×10^{-3} s^{-1} corresponds to a half-life of the LacI–DNA complex of 2.2 min in the 100 mM KCl binding buffer. A similar set of experiments was conducted for LacI in buffer in which KGlu was substituted for KCl. The rate of dissociation of LacI from a single site is, as expected from its increased equilibrium binding affinity, slower than that observed in KCl, with a half-life of 12.8 min (Table I).

Dissociation Kinetics of the Dimeric Lac^{adi} from a Single Specific Site. The deletion of the C-terminus of LacI results in the inability of the mutant proteins to associate from dimers to tetramers (Alberti et al., 1991; Chakerian et al., 1991). Lac^{adi} and other dimeric variants of LacI bind to specific sites, *in vitro*, with a reduced *apparent* affinity relative to the wild-type protein (Brenowitz et al., 1991b; Chen & Matthews, 1992). It was suggested by Brenowitz et al. (1991b) that the reduced apparent affinity is the result of a weakening of the monomer–dimer association reaction. The dissociation constant determined for the dissociation of Lac^{adi} is approximately 2-fold slower than the values determined for the wild-type protein (Table I, line 5). The dissociation data are well described by a single exponential (data not shown). However, comparison of the dissociation rates of wild-type and dimeric protein is potentially complicated by the differential dependence of the dissociation rate of LacI and Lac^{adi} on competitor concentration reported by Ruusala and Crothers (1992). Although the competitor concentrations used in this study are low relative to the concentrations at which Ruusala and Crothers observed significant facilitation of dissociation, the values determined for wild-type LacI should only be considered to the upper limits of the intrinsic dissociation rate; k_1 may overestimate the intrinsic dissociation constant severalfold at the competitor concentrations used. Comparable reductions in the dissociation rates of LacI and Lac^{adi} were observed upon the substitution of KGlu for KCl (Table I).

Dissociation Kinetics of the LacI-Mediated Looped Complex. The progress curves measured for the dissociation of LacI from DNA containing *two* identical sites separated by 11 helical turns were biphasic at even the lowest initial protein concentration of 5.0 pM (Figure 6). The biphasic progress curves are consistent with the presence of the looped complex and each of the "singly liganded forms" where the protein is bound to only one of the two sites.[3] A slow phase is absent in the progress curve for the dissociation of the dimeric Lac^{adi} from the DNA containing *two* specific sites. This result provides additional evidence that the slow phase corresponds to the dissociation of the looped complex, while the fast phase corresponds to the dissociation of protein from a single site (Figure 6, panel Lac^{adi}).

The progress curves shown in Figure 6 were analyzed as the sum of two exponentials.[4] In all cases, the values of k_1 are in reasonable agreement with the values of k_1 determined from the dissociation of LacI from a single site (Tables I and II). The values of k_2, corresponding to the looped complex, are appreciably slower. The ratio of the dissociation constants,

Table I: Dissociation of Lac Repressor Bound to DNA Containing a Single Specific Site[a]

[LacI], nM[b]	$k_1 \times 10^3$	σ[c]
	100 mM KCl	
0.05	5.2 (±1.0)	0.100
0.5	9.7 (±1.0)	0.055
3.9	8.3 (±1.4)	0.088
Lac^{adi} [d]	3.4 (±0.4)	0.033
	100 mM KGlu	
0.06	1.3 (±0.3)	0.101
Lac^{adi} [d]	1.1 (±0.4)	0.029

[a] First-order dissociation constants in units of s^{-1} determined for LacI–DNA complexes formed with limiting concentrations (≤1 pM) of a ^{32}P-labeled DNA restriction fragment contain a single *lac* recognition sequence. The buffer contained 100 mM KCl or KGlu at 20 °C (Materials and Methods). [b] Initial concentration of LacI expressed as nM dimer. [c] Square root of the variance of the fitted curves. [d] The initial concentration of Lac^{adi} is 10.0 nM.

[3] The biphasic nature of the dissociation progress curves of LacI mediated looped complexes has been noted previously as detected by nitrocellulose filter binding (Whitson & Matthews, 1986) and gel mobility-shift analysis (Eismann & Müller-Hill, 1990). Eisman and Müller-Hill (1990) ascribed the fast phase they observed in their dissociation studies to the degraded condition of their protein preparation, specifically, to a high proportion of their LacI protein being dimeric. Although we cannot comment on the purity of the protein preparations used in the studies of Eismann and Müller-Hill (1990), it is clear from these observations that the biphasic progress curves observed in our studies are characteristic of the dissociation of looped complexes. First, SDS–PAGE of the LacI preparation used in these studies reveals a single peptide of ~37 000 KDa (data not shown). Second, gel mobility-shift analysis of the LacI-mediated looped complex studied herein is also consistent with *all* the protein being competent to form a looped complex (Brenowitz et al., 1991a; unpublished data). Third, the dissociation progress curves of the dimeric Lac^{adi} bound to the DNA containing two sites are linear (Figure 6).

[4] The analysis of the progress curves for the dissociation of LacI from DNA containing two specific sites is potentially complicated by the fractional retention of LacI on nitrocellulose filters. The maximum retention efficiency of LacI bound to DNA containing one (O_E^G/O_1^L) and two sites (O_E^L/O_1^L) is ~60% and ~80%, respectively (data not shown). At LacI concentrations exceeding ~98% saturation, DNA containing two sites is predominantly in the form of "tandem complexes", i.e., one LacI tetramer is bound to each site under these experimental conditions (Brenowitz et al., 1991a). (The 80% retention of the tandem complex is in agreement with the 84% retention calculated from the independent probability theory; Woodbury & Von Hipple, 1983.) These experiments were conducted at sufficiently low LacI concentrations to minimize the contribution of tandem complexes and maximize the proportion of looped complexes present in the LacI–DNA mixture (Brenowitz et al., 1991a). The fact that equilibrium filter-binding titrations of LacI with the two-site DNA do not have anomalous shapes suggests that the looped complex has a retention comparable to the complex in which LacI is bound to only a single site (data not shown).

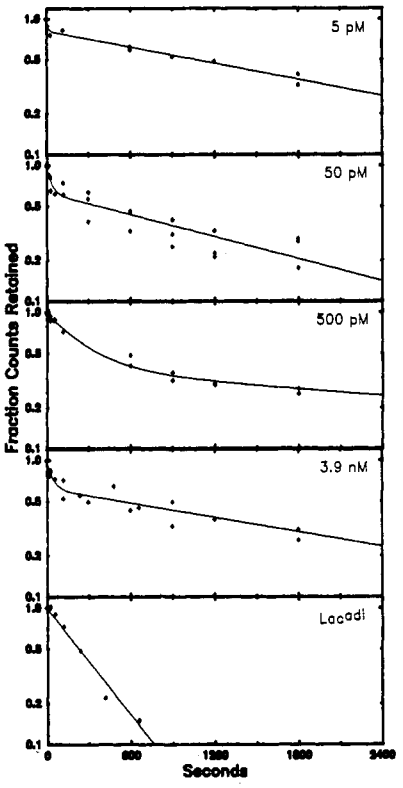

FIGURE 6: Dissociation progress curves for LacI bound to DNA containing two specific sites separated by 11 helical turns as a function of initial protein concentration. Data was collected until the fraction of the counts retained on the filter reached 10%. The plots in this figure were truncated at 300 s in order to more clearly represent the "fast component".

Table II: Dissociation of LacI Bound to DNA Containing Two "In Phase" Specific Sites[a]

[LacI], nM[b]	$k_1 \times 10^2$	$k_2 \times 10^4$	k_1/k_2	$\%k_1$[c]	σ[d]
		100 mM KCl			
0.005	1.0 (±1.3)	4.6 (±1.5)	21.7	18.8 (±5.0)	0.034
0.05	1.7 (±2.6)	6.3 (±2.0)	27.0	37.0 (±9.0)	0.079
0.5	0.39 (±0.12)	1.9 (±0.9)	18.9	61.7 (±8.0)	0.036
3.9	1.3 (±1.3)	4.1 (±1.5)	31.7	38.0 (±7.0)	0.044
Lac[adi][e]	0.8 (±0.2)				0.054
		100 mM KGlu			
0.05	0.13 (±0.04)	2.4 (±4.0)	5.4	39.1 (±13.2)	0.043

[a] First-order dissociation constants in units of s^{-1} determined for LacI–DNA complexes formed with limiting concentrations of ^{32}P-labeled DNA (≤1 pM) at the LacI concentrations indicated by analysis of the progress as the sum of two exponentials (see Materials and Methods). The DNA restriction fragments contain two *lac* recognition sequences separated by 11 helical turns (see Materials and Methods). [b] The total concentration of LacI is expressed in units of protein dimer. [c] Fraction of the progress curve assigned to k_1. [d] Square root of the variance of the fitted curves. [e] Lac[adi] at a concentration of 10.0 nM (units of dimer).

k_1/k_2, relates the relative stability of the "singly bound" and looped complexes. This ratio is equivalent to the isomerization constant, k_{iso} ($\Delta G°_{iso} = -RT \ln k_{iso}$), calculated from the footprint titration experiments. The value of k_{iso} of 37.1 determined under these experimental conditions is in reasonable agreement with the average with the averge value of k_1/k_2 of 24.5 calculated from the four determinations shown in Table II. When KGlu is substituted for KCl, the progress curves for the dissociation of LacI from the two-site DNA are also biphasic (data not shown). The k_1/k_2 ratio of 5.4 is in reasonable agreement with the value of k_{iso} of 7.9 determined from the footprint titration experiments (Table II).

DISCUSSION

LacI binds to specific DNA sequences with up to 6 or 7 orders of magnitude greater affinity than "nonspecific" DNA sequences. This binding is exquisitely sensitive to a number of thermodynamic variables, including temperature and ion type and composition [cf. Mossing and Record (1985), Ha et al. (1989, 1992) and Chakerian and Matthews (1988)]. The results presented in this paper show that the stability of a LacI-mediated looped complex can be regulated independently from the constituent protein–DNA interactions. Thus, the physical properties and topology of the DNA, the allosteric interactions within the loop-mediating protein, the relative geometry of the two DNA-binding contact regions, and the dimer–tetramer association are all potential sites for biological regulation. The differences in the response of these levels of interactions to biological effectors could be exploited to finely balance the occupancy of regulatory sites and the formation of a regulatory complex. Given the ubiquitous occurrence of protein-mediated DNA looping in regulation, it is essential to understand the contributions of each of these interactions to the process.

The affinity of LacI for the 16-bp symmetric sequence utilized in these studies is approximately 25-fold lower than its affinity for the symmetric sequence (O^{sym}) that includes 20 bp (Ha et al., 1989, 1992). The 20-bp sequence is

10 9 8 7 6 5 4 3 2 1
AATTGTGAGC·GCTCACAATT

The underlined base pairs are TT and TA in O_E^L and CG and TG in O_I^L, respectively. *In vivo* analysis of symmetric substitutions of T and G at position 9 suggest reductions in affinity of approximately 7- and 4-fold, respectively (Lehming et al., 1987). The changes measured *in vivo* are small, although it has been pointed out by Mossing and Record (1985) that reductions in affinity due to *lac* operator substitutions are reduced *in vivo* compared with studies conducted *in vitro*. The fact that O_E^L and O_I^L differ in affinity for LacI by less than 0.2 kcal/mol (Brenowitz et al., 1991a) suggests that any deviation from the symmetric sequence in the outer two base pairs results in a significant reduction in affinity. Alternatively, compensating base-pair changes more distant from the two-fold symmetry axis also influence binding.

In addition to the differences in affinity, the net number of ions released upon the formation of the protein–DNA complex is 8.2 ± 0.2 for the 20-bp sequence (Ha et al., 1992) compared with the value of 4.9 ± 0.2 determined herein for the 16-bp sequence. These results suggest that the contribution of the specific interactions made by the outside two base pairs is primarily electrostatic in nature. This conclusion is supported by the observations that the affinity and the salt dependence of the binding of LacI to operator are sensitive to the presence of flanking DNA sequences (O'Gorman et al., 1980; Whitson & Matthews, 1986). The systematic *in vitro* analysis of LacI binding to operators containing substitutions of base pairs 9 and 10 will be required to more clearly reveal their contribution to site-specific recognition. It is tempting to speculate that the contacts made by base pairs 9 and 10 contribute to the ability of LacI to bend the DNA to which it is bound (Zwieb et al., 1989). If so, the stability of a LacI-mediated looped complex utilizing the 20-bp sequence might differ from that predicted for the increase in $\Delta G°_{intrinsic}$ due to changes in the geometry of the interaction of the protein with the DNA.

Information concerning the flexibility of DNA has come predominantly from electrooptic and ligase-catalyzed cyclization studies [cf. Hagerman (1988)]. A potential concern

with use of DNase I footprinting to study the flexibility of DNA is the introduction during the assay of single-stranded nicks by DNase I. However, footprint titration experiments are conducted under conditions of "single-hit" kinetics, where each nicked molecule is nicked only once (Brenowitz et al., 1986), precluding perturbation of the protein–DNA equilibria. In addition, viscometric and sedimentation studies (Hays & Zimm, 1970) and cyclization studies (Shore & Baldwin, 1983a,b) indicate that the introduction of a small number of single-stranded nicks does not affect the flexibility of the DNA.

The cyclization free energy of the LacI-mediated looped complex studied herein of 11.7 kcal/mol is greater than the 6.5–7.7 kcal/mol calculated for bending the DNA 180° (assuming a harmonic potential: $\Delta G°_{bend} = 0.5B(1/R_c)^2L$, where $B = 2 \times 10^{-19}$ erg·cm (Hagerman, 1988), $R_c = 60$–65 Å (Steitz, 1990), and $L = 384$ Å for bending across a 360° arc). Although this calculation is clearly a rough approximation, the large discrepancy between the two values suggests that a component in addition to DNA flexibility makes a significant contribution to the free energy of cyclization (ΔG_j) of the LacI-mediated looped complex. A DNA torsional component may contribute to the cyclization free energy despite the fact that the two binding sites are nominally "in phase" with each other since the size and orientation of the protein, as well as the crossover angle of the DNA, are contributory factors [cf. Hagerman and Ramadevi (1990)]. In addition, the estimate of the cyclization free energy from quantitative footprint titration studies (Brenowitz et al., 1991a) is subject to the assumed value of the dimer–tetramer association free energy as well as the relative DNA-binding characteristics of LacI dimers and tetramers. The linkage of chloride ion binding with $\Delta G°_j$ (discussed below) highlights the critical role played by these additional protein-dependent processes in determining the stability of the looped complex.

The observation that the isomerization, hence the stability, of the LacI-mediated looped complex tracks with the changes in the DNA-binding affinity of the protein as a function of temperature is the result of the invariance of $\Delta G°_j$ as a function of temperature (Figure 1C). The contribution of thermal energy represented by RT results in a ~22-fold change in the cyclization probability (k_j) over the temperature range studied. These results are consistent with transient electric birefringence studies in which no significant change with temperature was determined for the rotational relaxation times (Hagerman, 1981) and with sedimentation studies in which only a small decrease in the persistence length of DNA was observed between 5 and 49 °C (Gray & Hearst, 1968). The invariance of ΔG_j with temperature suggests that the DNA within the LacI-mediated looped complex does not assume an unusual conformation.

The enthalpy calculated from the van't Hoff analysis of the cyclization of the LacI-mediated looped complex (Figure 1D) is large and positive, resulting in an entropy that is small and positive (2.2 cal/deg, $\Delta G° = \Delta H° - T\Delta S°$). Reconciliation of these results with those expected for free DNA is hampered by the absence of direct studies of the temperature dependence of DNA cyclization. However, comparisons with other data are possible. (1) Several studies have reported a dependence of DNA flexibility on base composition (Hogan et al., 1983; Chen et al., 1985), although the interpretation of these results has been questioned (Hagerman, 1988). Chen et al. (1985) suggest that the sequence dependence of the flexibility of DNA is in turn dependent on base-pair stability, which is primarily enthalpic in nature (Crothers & Zimm, 1964). (2) DNA supercoiling has also been shown to be primarily enthalpic in nature (Seidl & Hinz, 1984), also showing a small positive entropy. Although DNA supercoiling incorporates both bending and torsional components, the "ends" of the DNA are constrained in both relaxed and supercoiled plasmids, unlike the looped complex. (3) Analysis of a premelting transition in poly(dA)·poly(dT), believed associated with the "unbending" of the DNA, also reveals a positive enthalpy (Herrera & Chaires, 1989; Chan et al., 1990). Calorimetric studies of "bent" $[d(GA_4T_4C)]_2$ and "normal" $[d(GT_4A_4C)]_2$ oligonucleotides also suggest that a positive enthalpy is associated with the unbending of bent DNA (Park & Breslauer, 1991). The values of $\Delta H°$/bp of 1.6–1.8 (kcal/mol)/bp calculated from these studies are significantly greater than the value of 0.11 (kcal/mol)/bp calculated for bending DNA within the LacI-mediated looped complex. It is not clear why the enthalpy change per base pair is so much lower for "bending straight DNA" within the looped complex compared with "unbending intrinsically bent DNA". It is tempting to speculate that one molecular origin of the dominant enthalpic component of cyclization that we observe is the incremental loss of base-stacking interactions in the DNA within the looped complex, resulting in greater solvent accessibility of the base pairs. Support for this idea is the observed periodic DNase I hypersensitivity in the DNA within the LacI-mediated looped complex (Brenowitz et al., 1991a; data not shown) resulting from the distortion of the DNA helix within the loop. However, further studies will be required to resolve this issue.

The entropies calculated for the "unbending" transitions of intrinsically bent DNA are large and positive ($\Delta S°$ = 66 and 53 cal/deg, respectively; Herrera & Chaires, 1989; Chan et al., 1990). Correspondingly, large negative entropy changes would be expected for the LacI-mediated looped complex resulting from the loss of entropy associated with constraining the ends of the DNA. The small positive entropy change calculated for LacI-mediated looped complex formation may result from compensatory transitions, such as changes in the conformation of the protein.

In vivo analysis of the distance and phase dependence of LacI-mediated looped complexes yielded a value of the apparent persistence length that is much shorter than that measured for purified DNA *in vitro* (Bellomy et al., 1988; Law et al., 1993). However, as noted by these authors, the binding of the DNA-bending protein CAP to its specific site within the interoperator region or the level of DNA supercoiling may affect the apparent persistence length determined *in vivo*. The binding of DNA-bending proteins to the DNA within a looped complex is known to stabilize or destabilize the complex, depending on the phasing of their binding site with the loop (Goodman & Nash, 1989; Lobell & Schleif, 1991).

The linkage of chloride ion binding to $\Delta G°_j$ presented in this paper suggests that additional variables can have significant effects on the stability of protein-mediated looped complexes and suggests that such processes may play a role in modulating the stability of looped complexes *in vivo*. The observation that the stability of the LacI-mediated looped complex decreased when glutamate, the physiologically dominant counter-anion present in the cytoplasm of *E. coli*. (Cayley et al., 1991), was substituted for chloride was surprising since increases in the DNA-binding affinity of LacI should have resulted in substantially increased looped complex stability if no other process was affected. The persistence length of DNA is insensitive to the type and concentration of the anion present in solution and is thus unlikely to be the source of looped complex destabilization [cf. Hagerman

(1988)]. In addition, the relative DNA-binding affinities of LacI and the dimeric Lac^{adi} do not change in KCl relative to KGlu, indicating that the *intrinsic* affinity of dimers and tetramers for DNA is not differentially affected by chloride and glutamate. When the titration data obtained in 100 mM KGlu are analyzed, assuming the value of $\Delta G°_j$ of 11.7 determined in 100 mM KCl, reasonable fits to the data are only obtained either by increased dissociation of LacI tetramers to dimers or by anticooperative DNA binding by the tetramer in KGlu- relative to KCl-containing solutions. Since the analysis of the concentration distribution of the DNase I hypersensitive bands located between the operators, which provides a measure of $\Delta G°_{tet}$, does not suggest destabilization of the dimer–tetramer association (Figure 4), we conclude that the binding of chloride induces 1.5 kcal/mol of positive cooperative into DNA binding by the LacI tetramer.

What is the mechanism by which Cl^- modulates this process? The effect of glutamate ions on DNA binding has been ascribed to it acting as an inert counter-ion, whereas chloride ions directly compete for the binding of DNA by the protein (Ha et al., 1992). Studies on the stability of tubulin led to the conclusion that glutamate induces "a strong preferential hydration of the proteins" (Arakawa & Timasheff, 1984), and Ha et al. (1992) have suggested, on the basis of this data, that glutamate does not bind to LacI. One speculation is that the single linked chloride ion binds to a specific site on the tetramer, stabilizing the liganded conformation. In the absence of the bound chloride ion, the conformation of the tetramer is less favorable for binding the second DNA site. These conformational differences might be reflected in the crossover angle of the bound DNA or in the LacI-induced DNA bending (Zwieb et al., 1989). If the binding of DNA by each dimer in the tetramer is coplanar, then an increase in the protein-induced DNA bend might result in steric or electrostatic interactions between the DNA strands as they pass through the protein.

A number of biological roles have been suggested for the formation of protein-mediated looped complexes. Studies by von Hippel and co-workers [cf. Berg et al. (1981)] proposed a kinetic mechanism termed "intersegment transfer" that involves "the rapid and direct transfer of repressor from one segment of a DNA molecule to another". Such a mechanism would enhance association rates beyond the limit of three-dimensional diffusion but would not necessarily result in a stable looped complex. A second mechanism is that protein-mediated looped complexes increase the local concentration of a protein at a "primary target site". In this view, the second (or third) protein-binding site(s) serve to provide a "reservoir" of protein in proximity to the target site [cf. Bellomy et al. (1988)]. A variation of this local concentration mechanism is that formation of the protein-mediated looped complex results in bringing together otherwise disparate regions of DNA containing other additional repression or activating elements on the same or on separate DNA molecules [cf. Wu et al. (1992)]. A third mechanism is that the protein-mediated looped complex itself serves as a regulatory element [cf. Adhya (1989)]. In this mechanism, neither protein-binding site overlaps the regulatory target, but the formation of the DNA loop either precludes binding of a protein (such as RNA polymerase) or prevents it from exerting its enzymatic activity. A common feature of all of these models is their dependence on the characteristics of binding of the "looping protein".

The use of a protein-mediated looped complex as a regulatory element itself differs from the other mechanisms in two important ways. The first difference is that since a variable number of bases can be accommodated within the DNA loop, it is possible for a protein-mediated looped complex to exert regulatory control over a number of other regulatory systems. In addition, the protein-mediated looped complex might "trap" the target proteins in an inactive protein–DNA conformation. Upon de-repression, all of the components necessary for biological activity would be "prebound" to the DNA. The relative concentrations of DNA-bound protein and protein-mediated looped complexes could be modulated by allosteric effectors of either the dimer–tetramer equilibrium or the allosteric interactions between the DNA-binding sites within the protein. Since the free energy of the looped complex is $2\Delta G°_{intrinsic} + \Delta G°_j$, any process that would make the cyclization free energy significantly more negative, such as the binding of a DNA-bending protein or DNA supercoiling, would result in a regulatory complex of extraordinary stability. For example, if $\Delta G°_j = 0$, then the stability of the looped complex is equal to $2\Delta G°_{intrinsic}$. If $\Delta G°_{intrinsic} = -14.0$ kcal/mol, then the K_d of the looped complex would be on the order of 10^{-21}. Such complexes would be essentially nondissociable and might serve to inactivate processes throughout the cell cycle. Indeed, LacI-mediated looped complexes formed on supercoiled DNA have long half-lives (Hsieh et al., 1987; Krämer et al., 1988). In such a system, the regulatory protein could remain bound to the DNA with the looped complex specifically stabilized or destabilized by an effector such as chloride. Thus, protein-mediated looped complexes provide a regulatory element of potentially high stability that can be controlled with a high degree of specificity.

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