**Ab initio** thermodynamic modeling of distal multisite transcription regulation

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**ABSTRACT**

Transcription regulation typically involves the binding of proteins over long distances on multiple DNA sites that are brought close to each other by the formation of DNA loops. The inherent complexity of assembling regulatory complexes on looped DNA challenges the understanding of even the simplest genetic systems, including the prototypical lac operon. Here we implement a scalable approach based on thermodynamic molecular properties to model **ab initio** systems regulated through multiple DNA sites with looping. We show that this approach applied to the lac operon accurately predicts the system behavior for a wide range of cellular conditions, which include the transcription rate over five orders of magnitude as a function of the repressor concentration for wild type and all seven combinations of deletions of three operators, as well as the observed induction curves for cells with and without active catabolite activator protein. Our results provide new insights into the detailed functioning of the lac operon and reveal an efficient avenue to incorporate the required underlying molecular complexity into fully predictive models of gene regulation.

**INTRODUCTION**

Control of localized events on DNA by proteins bound at distal sites is intimately linked to DNA looping. DNA flexibility plays an important role in mediating long-range interactions (1), allowing proteins bound to nonadjacent DNA sites to come close to each other. This strategy is widely used in eukaryotic enhancers (2) to integrate multiple signals into the control of the transcriptional machinery (2,3), to the extent that transcription regulation through DNA looping is nowadays considered to be the rule rather than the exception (4,5). DNA looping can also be formed by single-protein complexes, including the regulators of many bacterial operons, such as ara, gal, and lac operons (6), and human proteins involved in cancer, such as retinoic X receptor (RXR) (7) and p53 (8). The presence of DNA looping with single- and multi-protein complexes is important not only for transcription regulation but also for many other cellular processes, including DNA replication (9), recombination (10), nucleosome positioning (11), and telomere maintenance (12).

To date, there is only limited understanding of the factors that drive macromolecular assembly on looped DNA, especially when multiple binding sites and loops are involved (1,13). A notable example is the lac operon in *Escherichia coli*, which is still far from being completely understood despite being one of the systems that led to discovery of gene regulation (14).

The *E. coli* lac operon is the genetic system that regulates and produces the enzymes needed to metabolize lactose (14). The response to lactose is controlled by the lac repressor (15) that can bind to O1, the main operator, thus preventing the RNA polymerase from binding to the promoter and transcribing three genes used in lactose metabolism. There are also two auxiliary operators, O2 and O3, to which the repressor can also bind, but not prevent transcription (Figure 1). Elimination of either one auxiliary operator has only minor effects; yet simultaneous elimination of both of them reduces the repression level by a factor 100 (16). This effect results from the ability of the lac repressor, a tetramer with two dimeric DNA-binding domains, to bind simultaneously to two operators and loop the intervening DNA. Thus, the main operator and at least one auxiliary operator suffice to form DNA loops that substantially increase the ability of the repressor to bind the main operator. Beyond increasing the repression level, it is not clear to what extent DNA looping shapes the properties of transcription regulation and the effects that having three instead of two operators has on the behavior of the system. Recent phenomenological analyses of the lac operon (17,18) have been able to fit...
the approach of Ref. (19), we consider a decomposition of the free energy of the protein–DNA complex into positional, interaction, and conformational contributions. In short, the positional free energy, \( p \), accounts for the cost of bringing the \( lac \) repressor to its DNA-binding site in the protein–DNA complex; interaction free energies, \( e \), arise from the physical contact between the repressor DNA-binding domains and the different operator sites; and conformational free energies, \( c \), account for changes in conformation, including the formation of DNA loops (Figure 1). All these contributions to the free energy can be collected to obtain the free energy \( \Delta G(s) \) of a given state \( s \) of the protein–DNA complex. The advantage of this approach is that it provides the free energies of a large number of different states from just the individual properties of the interactions and components. Here, different states account for the different ways in which the repressors can bind the three operators.

The free energy of a state \( s \) is connected to the equilibrium probability \( P_s \) of such state through the statistical thermodynamics relationship \( P_s = \frac{1}{Z} e^{-\Delta G(s)/RT} \), where \( Z = \sum_s e^{-\Delta G(s)/RT} \) is the partition function, which serves as normalization factor, and \( RT \) is the gas constant times the absolute temperature (20).

Straightforward application of the traditional thermodynamic approach (21) in a general framework is of limited use because the number of states that must be considered typically increases exponentially with the number of components (13). For instance, just the binding of the \( lac \) repressor to three DNA sites would lead to eight states, including states with zero, one, two, and three repressors bound to DNA. If DNA looping is taken into account, the number of states increases to 14. It has become clear recently that it is possible to overcome this limitation and express the free energy of all these states in a compact form by using binary variables (13). By extending the approach of Ref. (13) to consider multiple loops in the \( lac \) operon, we obtain

\[
\Delta G(s) = (p + e_1)s_1 + (p + e_2)s_2 + (p + e_3)s_3 + (c_{L12} + c_{L13} + c_{L23})
\]

where \( s_1 \), \( s_2 \), and \( s_3 \) are binary variables that indicate whether \( s_i = 1 \), for \( i = 1,2,3 \) or not \( s_i = 0 \), for \( i = 1,2,3 \) a repressor is bound to \( O_1 \), \( O_2 \), and \( O_3 \), respectively; and \( s_{L12}, s_{L13}, s_{L23} \) are binary variables that indicate whether \( s_{Lij} = 1 \), for \( i = 1,2,3 \) or not \( s_{Lij} = 0 \), for \( i = 1,2,3 \) DNA forms the loops \( O_1-O_2 \), \( O_1-O_3 \), and \( O_2-O_3 \), respectively. For instance, using the notation \( s \equiv (s_1, s_2, s_3, s_{L12}, s_{L13}, s_{L23}) \) the state shown in Figure 1B is specified by \( s = (1,1,0,1,0,0) \) and that of Figure 1C, by \( s = (1,1,0,1,0,0) \). The subscripts of the different contributions to the free energy have the same meaning as those of the corresponding binary variables. In this case, with three interaction and three conformational free energies, it is possible to obtain the free energy of 14 states for different repressor concentrations. The dependence on the repressor concentration, \( n \), enters the free energy through the

**Figure 1.** Operators controlling expression of the \( lacZ \), \( lacY \), and \( lacA \) genes in the \( lac \) operon. (A) Location of the main \( (O_1) \) and the two auxiliary \( (O_2 \) and \( O_3) \) operators, shown as orange rectangles on the thick black segment representing DNA. Binding of the \( lac \) repressor to \( O_1 \) prevents transcription of the three \( lacZA \) genes. (B, C) The bidentate repressor can bind to any of the three operators and simultaneously to any two of them by looping the intervening DNA, resulting in different protein–DNA complexes. Two of the three possible loops are shown: (B) one \( lac \) repressor (shown in red) loops DNA by binding simultaneously to \( O_1 \) and \( O_3 \) (loop \( L13 \)) whereas another repressor binds to \( O_2 \); (C) only one repressor is bound to DNA, forming a loop between \( O_1 \) and \( O_2 \) (loop \( L12 \)). In both cases, the \( lacZA \) genes are repressed. The different contributions to the free energy of the \( lac \) repressor–DNA complexes, which include positional \( (p) \), interaction \( (e_1, e_2, \) and \( e_3) \), and conformational \( (c_{L12}, c_{L13}, \) and \( c_{L23}) \) contributions, are explicitly indicated in these two cases.

**METHODS**

To incorporate the relevant molecular properties into a quantitative model of transcription regulation, we have used a statistical thermodynamics approach. Following
positional free energy: \( p = p^o - RT \ln n \), where \( p^o \) is the positional free energy at 1 M concentration. An important advantage of the binary variable description is that it can straightforwardly implement ‘logical conditions’. For instance, the infinity in the last term of the free energy implements that two loops that share one operator cannot be present simultaneously by assigning an infinite free energy to those states.

The experimental observations, as summarized in Ref. (22), indicate that in the lac operon, transcription is completely abolished when the repressor is bound to O1. If O1 is free, transcription takes place either at an activated maximum rate \( \tau_{\text{max}} \) when O3 is free or at basal reduced rate \( \chi \tau_{\text{max}} \) when O3 is occupied. The reduction from the activated state by a factor \( \chi \) arises because binding of the repressor to O3 prevents CAP, also known as cAMP Receptor Protein (CRP), from contacting the RNA polymerase and activating transcription (22). Activation is achieved when CAP bound to cyclic AMP (cAMP) binds between O3 and O1, contacts the RNA polymerase, and stabilizes its binding to the promoter (23,24). All these experimental facts can be accounted for by the transcription rate \( \tau(s) \) expressed in terms of binary variables as

\[
\tau(s) = \tau_{\text{max}}(1-s_1)(\chi s_3 + 1 - s_3),
\]

which provides a mathematical expression for the observed transcription control (22,25).

The concise mathematical expressions \( \Delta G(s) \) and \( \tau(s) \) completely specify the thermodynamic and transcriptional properties of the system. In particular, the repression level, defined as the maximum transcription over the actual average transcription rate, is given by \( R = \tau_{\text{max}}/\bar{\tau} \), where the average transcription rate follows from \( \bar{\tau} = \frac{1}{2} \sum s_i \tau(s) e^{-\Delta G(s)/RT} \). Thus, with this approach, it is possible to obtain a compact description for both the DNA–repressor complex and the control of transcription that scales linearly with the number of regulatory elements (13).

RESULTS

Our model accurately reproduces the observed behavior of the lac operon (22) in quantitative detail over five orders of magnitude of the repression level for three repressor concentrations and eight strains with all the possible combinations of operator deletions (Figure 2). The values of the parameters used are the same as those previously reported (1) except for two small variations. One of the changes is a 0.35 kcal/mol shift in the affinity of the repressor for the operators. This difference falls within the typical variation for different experimental conditions. The other change is a decrease of 0.9 kcal/mol in the free energy of forming the O1–O2 loop. This decrease is consistent with stabilization of the loop by binding of CAP to its DNA site between O1 and O2, which has been estimated to be within –0.8 kcal/mol and –1.4 kcal/mol with an average value of –1.1 kcal/mol (26) and is similar to the values obtained for other proteins that bend DNA, such as the HU protein (27). Note that the experiments used to infer the free energies of looping (28,29) were performed with artificial constructs that did not contain the CAP-binding site within the loop. We have also observed that the experimental repression levels are better accounted for if the deletion of O1 is not considered to be complete. Explicitly, we infer that the binding of the repressor is not completely abolished but reduced by \(~5\) kcal/mol, which is consistent with the mutation of just three base pairs of the operator (22). This strong reduction of affinity would be virtually indistinguishable from a complete deletion in a single operator setup, but it is not so in a multi-loop configuration.
The approach we have developed can straightforwardly be extended to study the induction curves of the lac operon, which provide the steady-state transcription rate as a function of the inducer concentration. Inducers, such as allolactose in the natural environment and isopropyl-β-D-thiogalactoside (IPTG) in typical experimental setups, bind to the repressor and impair its function (Figure 3). Specifically, each of the four identical monomeric components of the repressor can bind independently an inducer molecule. Each of the two dimers that form the tetrameric lac repressor has a DNA-binding domain. Binding of either one or two inducer molecules to a dimeric unit strongly reduces its affinity for operator DNA. Therefore, with one or two inducer molecules in the same dimeric domain, the repressor can bind strongly to one operator but cannot loop DNA; with two or more inducer molecules in different domains, strong operator binding is also abolished (Figure 3).

Our model accurately reproduces the observed in vivo induction properties of the lac operon when we consider as functional repressors only those that do not have any inducer bound. This approximation is very good because the simultaneous binding of a repressor to two operators on looped DNA is much stronger (~600 times) than the binding to a single operator and because the repressor concentration is very low, about ten repressors per cell (see below for a more general treatment). Thus, the concentration of functional repressors is given by \( n = n_T P_F \), where \( n_T \) is the total repressor concentration, \( P_F \) is the probability that a monomer of the tetrameric repressor is not bound to IPTG, and \( P_F \) is the probability that no IPTG molecule is bound to the repressor. The probability for a repressor monomer to be free of IPTG follows from \( P_F = \frac{1}{1 + [I]/K_i} \), where \([I]\) is the intracellular IPTG concentration and \( K_i \) is the inducer-repressor equilibrium dissociation constant (30). It is important to note that in vitro studies have found that IPTG can bind cooperatively when the operator DNA concentration is at least 100 nM (31). In vivo, however, as in the experiments considered here, such high operator concentrations are never reached (one operator per E. coli cell corresponds to a concentration of ~1.5 nM).

For the reported value of \( K_i \) (30) and the previous values of the parameters, without any free parameter, our model reproduces exactly the experimentally observed induction curves (17) of cells with active CAP (Figure 4A; lower dashed red curve labeled ‘CAP’). The model, however, does not accurately reproduce the experimental data (Figure 4A; upper dashed red curve labeled ‘No CAP’) for induction of the lac operon without active CAP. We modeled this condition with the inferred value of the free energy of forming the O1-O3 loop in the absence of active CAP and with \( \tau(s) = \frac{s}{2} \sum \tau_{max} X (1 - s) e^{-\Delta G_0/RT} \), which considers that there is no activated transcription.
Previous studies based on phenomenological models have shown that, it is possible to fit the experimental results by assuming that the absence of active CAP reduces the effects of looping in repression by a factor \( \sim 10 \) (17). However, such a factor appears to be extremely high because half of the contribution to repression comes from the \( O_1-O_2 \) loop, which is not affected by CAP. Therefore, within the current molecular view of the \( lac \) operon, the absence of CAP could only reduce the effects of looping by a factor \( \sim 2 \) at most. Another possibility not considered so far is to assume a different repressor concentration, which has been found to depend on the binding of CAP in the regulatory region through the post-transcriptional effects of the SsrA system (32).

In the general case that accounts for arbitrary repressor concentrations, we need to consider also the concentration of repressors with one, \( n_d = n_T(4P_T^2(1-P_T) + 2P_T^2(1-P_T)^2) \), and two, \( n_i = n_T - n - n_d \), inducer-impaired DNA-binding domains (Figure 3). The concentration \( n_t \) needs to be considered explicitly because the inducer substantially reduces the binding of the repressor to operator DNA but it does not abolish it completely (33). This ‘residual’ binding is taken into account by an offset \( r \) in the free energy of interaction with the operators. The expression of the free energy for the binding of the three repressor types is given by

\[
\Delta G_T(r) = \Delta G(s) + (p_{d} + e_{i})s_{1d} + (p_{d} + e_{i})s_{2d} + (p_{d} + e_{i})s_{3d} + (p_{d} + e_{i} + r)s_{1i} + (p_{i} + e_{i} + r)s_{2i} + (p_{i} + e_{i} + r)s_{3i} + \infty(s_{1r}s_{1d} + s_{2r}s_{2d} + s_{3r}s_{3d} + s_{1r}s_{1i} + s_{2r}s_{2i} + s_{3r}s_{3i} + s_{1d}s_{1i} + s_{2d}s_{2i} + s_{3d}s_{3i}),
\]

where \( d \) and \( i \) subscripts refer to the binding of repressors with one and two impaired DNA-binding domains, respectively. The corresponding positional free energies are given by \( p_{d} = p^0 - RT\ln n_d + RT\ln 2 \) and \( p_{i} = p^0 - RT\ln n_i \). The term \( RT\ln 2 \) in the expression of \( p_d \) takes into account that only one of the two DNA-binding domains is available for binding. The infinity in the last term implements that an operator cannot be occupied simultaneously by more than one repressor, which can be a repressor with zero, one, or two inducer-impaired DNA-binding domains. Note that other possible states, such as those accounting for looping of a repressor with inducer bound, are not taken into account because they are energetically very unfavorable for the observed large values of \( r \).

Now, \( s \equiv \{s_{1r}, s_{2r}, s_{3r}, s_{1i1}, s_{1i2}, s_{2i1}, s_{2i2}, s_{3d1}, s_{3d2}, s_{3d3}, s_{1i}, s_{2i}, s_{3i}\} \) specifies the state of the protein–DNA complex through the values of 12 binary variables. The number of states has increased from 14 to 76 but the only additional parameter is \( r \), which has been measured experimentally \( (r = 2.8\text{ kcal/mol}) \) using operator fragment binding analysis (33).

The control of transcription is as follows: occupancy of \( O_1 \) by any repressor type always prevents transcription, and occupancy of \( O_3 \) prevents activated transcription when active CAP is present (if active CAP is absent, there is never activated transcription). Therefore, the mathematical expression of the transcription rate in the presence of active CAP is

\[
\tau_T(r) = \tau_{max}(1 - (s_1 + s_1d + s_1i))
\]

\[
(\chi(s_3 + s_3d + s_3i) + 1 - (s_3 + s_3d + s_3i))
\]

and in the absence of active CAP is

\[
\tau_T(r) = \tau_{max}(1 - (s_1 + s_1d + s_1i)).
\]

Note that these expressions use the fact that only one of the variables \( s_{1r}, s_{1r}d, s_{1i} \) and only one of the variables \( s_{3r}, s_{3r}d, s_{3i} \) can be one at a given time.

The experimental data in the absence of active CAP is reproduced in detail just by setting the repressor concentration to 300 nM, equivalent to 200 repressors per cell (Figure 4B; upper full red curve labeled ‘No CAP’). This repressor concentration is 10 times higher than that of wild type with active CAP but 4.5 times smaller than that of the mutants studied in Ref. (22) that express 900 repressors per cell. In the presence of active CAP, the results obtained are in excellent agreement with both the experimental data (Figure 4B; lower full red curve labeled ‘CAP’) and the low-repressor concentration model (Figure 4A; lower dashed red curve labeled ‘CAP’). Thus, the general model accurately reproduces the experimental observations in the whole range of IPTG concentrations and cellular conditions with and without active CAP.

**DISCUSSION**

Regulation of gene expression by the assembly of protein–DNA complexes is strongly dependent on networks of molecular interactions that extend beyond simple binding events. A key issue is, therefore, to understand how the underlying molecular complexity shapes gene expression and controls the cellular behavior. Here we have shown that, in the case of the \( lac \) operon, incorporation of the molecular details—such as the presence of multiple DNA loops, the stabilization of loops by DNA-binding proteins, and residual binding of inducer-inactivated repressors and deleted sites—is crucial for the development of fully predictive models. Our results illustrate how to accurately infer the effects of multiple distal DNA-binding sites from the thermodynamic properties of the molecular components and provide new insights into the detailed functioning of the \( lac \) operon, including the role of CAP and the effects of the inducer.

Two key features of the thermodynamic approach we have implemented are the decomposition of the free energy of protein–DNA complexes into different modular contributions and the description of the different molecular states through binary variables. These two features taken together lead to concise mathematical expressions that completely specify the thermodynamic and transcriptional properties of all the states of the protein–DNA complex from just a few molecular parameters. In this way, we are able to circumvent one of the major issues that have precluded the development of computational approaches to study complex gene regulation systems;
nearly, the individual specification of the properties of all the potential states of the complex, which typically results in large itemized lists that increase exponentially with the number of interactions and components (13,34,35). Here we have shown explicitly with the lac operon that our approach can successfully be scaled up to include multiple molecular species, binding sites, and DNA loops.

Fully predictive computational approaches able to efficiently address multiple complex interactions, like the one we have implemented here, are becoming increasingly important not only for understanding gene regulation in natural systems but also for designing synthetic genetic networks with multiple components (36,37). This type of computational approaches is especially important because complex systems can reveal the effects of molecular details that were hidden in simpler setups used to characterize the components (38).

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