606–612 *Nucleic Acids Research, 2006, Vol. 34, No. 2*
doi:10.1093/nar/gkj453

Induction of the *lac* promoter in the absence of DNA loops and the stoichiometry of induction

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Received as resubmission October 22, 2005; Revised November 11, 2005; Accepted January 4, 2006

ABSTRACT

***In vivo* induction of the *Escherichia coli* lactose operon as a function of inducer concentration generates a sigmoidal curve, indicating a non-linear response. Suggested explanations for this dependence include a 2:1 inducer–repressor stoichiometry of induction, which is the currently accepted view. It is, however, known for decades that, *in vitro*, operator binding as a function of inducer concentration is not sigmoidal. This discrepancy between *in vivo* and *in vitro* data has so far not been resolved. We demonstrate that the *in vivo* non-linearity of induction is due to cooperative repression of the wild-type *lac* operon through DNA loop formation. In the absence of DNA loops, *in vivo* induction curves are hyperbolic. In the light of this result, we re-address the question of functional molecular inducer–repressor stoichiometry in induction of the lac operon.**

INTRODUCTION

The lactose operon of *Escherichia coli* is, together with phage lambda (1), probably the best analysed model system for transcriptional regulation (2). The interactions of Lac repressor with *lac* operator and inducer have been the subject of intensive studies for about five decades. The culmination so far of these efforts has been the solution of X-ray crystal structures of repressor bound to *lac* operator, non-operator DNA or to the gratuitous inducer isopropyl-β,D-thiogalactoside (IPTG) (3–5). Still, important features of regulation in the *lac* operon have yet to be fully elucidated.

It had early been noticed that *in vivo* expression of the *lac* operon as a function of inducer concentration does not follow a simple hyperbolic saturation function but instead yields a distinctively sigmoidal curve (6). This observation has prompted over the years several different explanations: cooperative inducer binding, a two-step mechanism of induction and the suggestion that two molecules of inducer are necessary to abolish operator binding of Lac repressor (7–11). The latter assumption prevailed and became the accepted view (12,13), despite the fact that *in vitro* binding studies of Lac repressor binding to *lac* operator, later tacitly ignored, failed to show the non-linearity of *in vivo* induction (14). It should be pointed out that most of these studies have been performed before it was generally realized that Lac repressor can bind to two operator sequences at the same time (15,16).

Lac repressor is a homo-tetramer that can be thought of as consisting of two DNA-binding dimers (9,17) which aggregate into a dimer of dimers by means of a 4-helix bundle (3,5,18,19). While a Lac repressor dimer is needed for specific DNA binding, each monomer binds with equal affinity to one molecule of inducer (20,21).

Lac repressor is the negative regulator of the lactose operon (22). The repressor prevents initiation of transcription of the *lac* messenger RNA by binding with high affinity to the first *lac* operator, O_1 (23), which lies immediately downstream of the *lac* promoter. Occupancy of O_1 by Lac repressor is cooperatively increased through DNA loop formation (24) by binding of the other dimer of the homotetrameric Lac repressor to either of the auxiliary operators, O_2 (25), which lies downstream of the *lac* promoter within the coding sequence of *lacZ*, or O_3 (26), which lies upstream of the *lac* promoter.

Upon binding of inducer, the conformation of Lac repressor changes such that the DNA-binding headpieces alter their orientation relative to the repressor core and relative to each other (5). As a result, affinity to operator drops ~1000-fold (27), and transcription of the *lac* messenger RNA increases accordingly. The induction of the *lac* operon as a function of inducer concentration can be followed *in vivo* through the expression of β-galactosidase, which is encoded by the first gene (*lacZ*) of the tricistronic *lac* mRNA. These measurements are done in a Lac permease negative background ($lacY^-$), where the cellular concentration

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of the gratuitous inducer IPTG is close to that of the medium (28).

Our earlier finding of cooperativity in repression of the *lac* operon (24) prompted us to look into the difference between *in vivo* and *in vitro* induction curves and to re-evaluate the current model for induction of the *lac* operon.

MATERIALS AND METHODS

Strains, lambda phages and plasmids

E.coli strain BMH 8117 is Δ(*lac*, *proAB*). *E.coli* strains BMH 8117(λEwt100) and BMH 8117(λEwt123) are derivatives of BMH 8117 which carry lambda prophages bearing the β-galactosidase gene under control of the indicated combination of *lac* operators (24). Wild-type tetrameric Lac repressor and dimeric active Lac repressor were expressed from plasmids pSO1010-P1 and pSO331Stop (carrying an i^{adi} allele), (24,29), respectively, for *in vivo* repression measurements. Constitutive expression was determined in the presence of plasmid pSO1000ΔA (24), bearing an i^- allele. Plasmid pWB1000 (30) was used for over-expression of wt tetrameric Lac repressor for *in vitro* experiments.

Plasmid pBlueOid was generated by cloning the ideal *lac* operator, O_{id}, which has an ∼10-fold higher affinity to Lac repressor than O_1 (31,32), from pWB300 (33) as a XbaI fragment into the SpeI site of pBluescript SK(+) (Stratagene). Plasmid pBCOid(−2) was constructed by cloning the polylinker, containing the ideal *lac* operator, from pBlueOid as a KpnI–SacI fragment into the respective sites of pBC KS(+), followed by deletion of 4 bp (blunting of the PstI site) and insertion of 2 bp (fill-in of the ClaI site).

β-Galactosidase assays

Specific β-galactosidase activities were determined as described (24,34). Each data point is the mean from two experiments. Constitutive expression is the mean of at least five independent cultures.

Band shift assays

Binding of wt Lac repressor to a single *lac* operator *in vitro* as a function of IPTG concentration was determined by band shifts, which were performed as described (35). Binding reactions with a 257 bp radiolabelled DNA fragment carrying a single ideal *lac* operator at 5×10^{-11}M and with 2×10^{-10}M Lac repressor dimer had a volume of 20 μl. The DNA fragment was generated by PCR, using primers PCRfor1 (5′-GTTGT AAAACGACGGCC-3′), PCRrev1 (5′-CAGGAAACAGCTA TGACC-3′) and plasmid pBlueOid as template. Non-bindable DNA was estimated as unbound DNA at 2.2×10^{-9} M Lac repressor dimer and corrected for.

Band shifts of wt Lac repressor binding to a DNA fragment with two *lac* operators (ideal operator O_{id} and wt O_1) were as above, with the following modifications: a 298 bp DNA fragment was generated using primers PCRfor1 and PCRrev0 (5′-GCTCGTATGTTGTGTGG-3′) and plasmid pBCOid(−2) as template. Binding reactions were separated on 4% acrylamide gels (acrylamide to *bis*-acrylamide, 79:1).

Lac repressor was partially purified with a 40–50% ammonium sulfate cut from sonication extracts in CSB (36) of BMH 8117 cells carrying pWB1000. Concentration of active repressor was determined by stoichiometric titration with 5×10^{-10} M ideal *lac* operator fragment. Dried gels were analysed with a Storm 840 phosphoimager (Molecular Dynamics). Phosphoimager data were quantified with ImageQuant 5.2 (Molecular Dynamics). Data points are the means of at least five experiments.

Data analysis

To derive operator binding data from specific β-galactosidase activities, we substituted:

$[O_t]$ (total operator) with A_c,
$[O_f]$ (free operator) with A,
$[O_{oc}]$ (occupied operator) with $A_c - A$,

where A_c = constitutive specific β-galactosidase activity and A = specific β-galactosidase activity at a given concentration of IPTG.

Regression analyses were performed with the program DataFit 7.0 (Engeneered Software, PA).

RESULTS

Induction of the *lac* promoter in the presence and in the absence of DNA loops

We first compared the *in vivo* induction curve of the wild-type (wt) configuration of the *lac* operon (the *lac* promoter is controlled through DNA loops by all three wt *lac* operators and wt tetrameric Lac repressor) with an *in vitro* induction curve of wt Lac repressor binding to a DNA fragment containing a single *lac* operator (Figure 1A and B). The graphs reflect the known discrepancy: The *in vivo* curve is sigmoidal, the *in vitro* curve is hyperbolic.

We then determined two *in vivo* induction curves of *lac* promoters controlled without DNA loops. Figure 1C: in the presence of wt tetrameric Lac repressor but only the first *lac* operator. Figure 1D: in the presence of all three *lac* operators but repressed by a mutant dimeric active Lac repressor. Neither combination allows DNA loop formation (24). Both curves are hyperbolic at inducer concentrations above the dissociation constant ($\sim 5 \times 10^{-6}$ M) of the repressor–IPTG complex (37,38), demonstrating that the sigmoidality of the induction curve of the wt system reflects cooperative repression through DNA loop formation. Consequently, induction data of the wt *lac* promoter do not allow straightforward inference as to the functional stoichiometry of inducer–Lac repressor dimer interaction. To address the question of how many molecules of inducer are required to abolish operator binding of a Lac repressor dimer, systems without DNA loops have to be analysed.

Figure 2 demonstrates that, also *in vitro*, template binding of Lac repressor engaged in a DNA loop, plotted as a function of inducer concentration, yields a sigmoidal induction curve. Lac repressor forms DNA loops *in vitro* with a DNA template carrying two suitably spaced operators, here O_1 and the ideal *lac* operator O_{id}, with the centres of symmetry separated by 168 bp, corresponding to 16 helical turns. Loop complexes with a linear template are less stable than those that can be achieved with supercoiled templates (39). Also, in contrast to

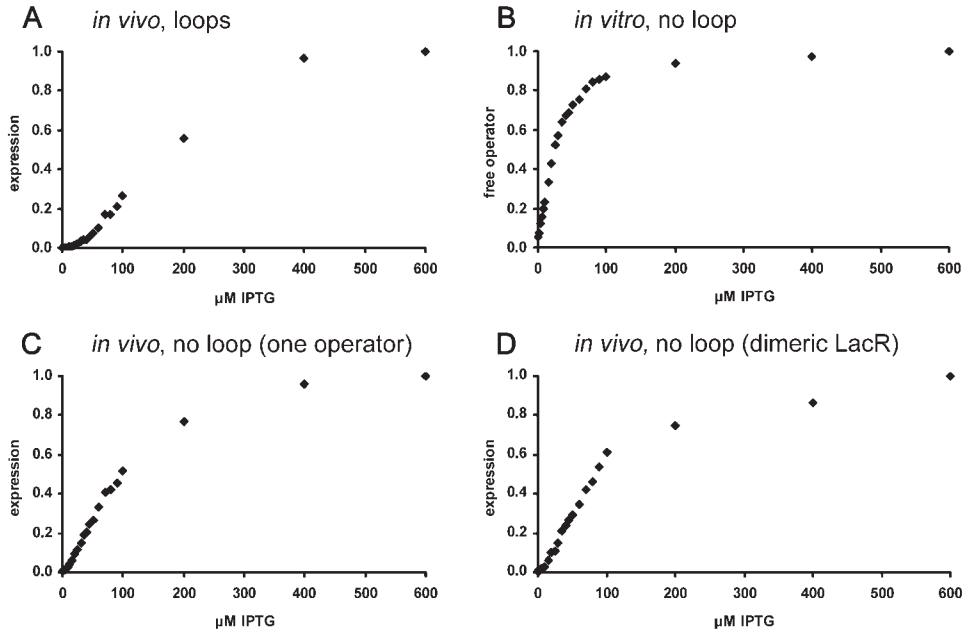

Figure 1. Induction of cooperative and non-cooperative *lac* systems. (**A**) Relative β-galactosidase activities (corresponding to a plot of $[O_f]/[O_t]$) of strain BMH 8117 (*lacY*$^-$), harbouring a plasmid expressing wt Lac repressor and carrying on a λ prophage a *lacZ* gene driven by the wt *lac* promoter with all three operators, are plotted against the IPTG concentration in the growth medium. (**B**) Complex of wt Lac repressor with a DNA fragment carrying a single *lac* operator. The fraction ($[O_f]/[O_t]$) of free DNA fragment (total fragment concentration is 5×10^{-11} M) in the presence of 2×10^{-10} M wt Lac repressor is plotted as a function of IPTG concentration. (**C**) As in (A), but in the presence of the first *lac* operator only. (**D**) As in (A), but harbouring a plasmid expressing dimeric active Lac repressor instead of wt tetrameric Lac repressor. To normalize the curves, values at 600 μM IPTG are set to 1.

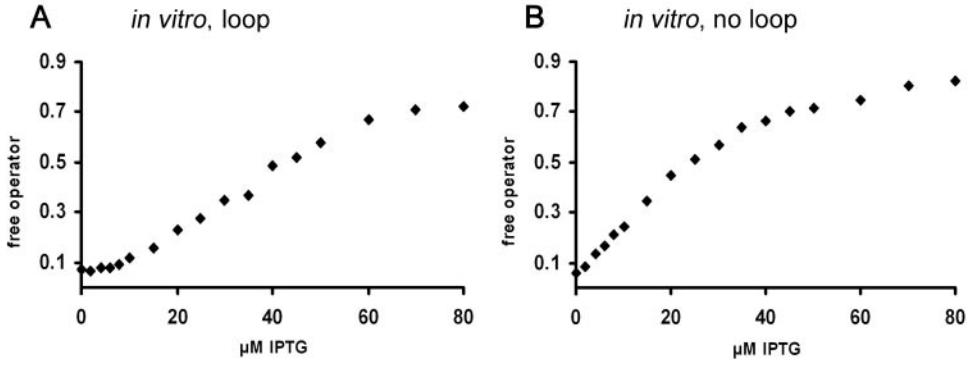

Figure 2. Induction of a DNA loop *in vitro*. The fraction ($[O_f]/[O_t]$) of free DNA fragment (total fragment concentration is 5×10^{-11} M) in the presence of 2×10^{-10} M wt Lac repressor is plotted as a function of IPTG concentration. Note the different scale compared with Figure 1. (**A**) Loop complex of wt Lac repressor with a DNA fragment carrying two *lac* operators separated by 16 helical turns. (**B**) Control: complex of wt Lac repressor with a DNA fragment carrying a single *lac* operator. To normalize the curves, values at 600 μM IPTG are set to 1.

the *in vitro* conditions, loops *in vivo* appear to be additionally stabilized by architectural DNA-binding proteins which increase the flexibility of the DNA (40). Cooperative binding of Lac repressor *in vitro* is accordingly weaker than *in vivo* and the sigmoidality of the induction curve restricted to its initial part (Figure 2A).

The stoichiometry of *lac* induction

In an experimental system devoid of DNA loops, three possible forms of the Lac repressor dimer, *D* (free dimer), *DI* (complex of dimer with one molecule of inducer) and DI_2 (complex of dimer with two molecules of inducer) can react with *lac* operator. In our experiments, inducer is in a large excess over repressor, and two identical and independent inducer-binding sites can be assumed for a Lac repressor dimer (20,21). Expressing the respective Lac repressor–inducer complexes as the fraction of total repressor $[D_t]$, the occupancy *R* (defined as the quotient $[O_{oc}]/[O_f]$ of operator O_{oc}, occupied by any one of the three forms of the repressor dimer, and free operator O_f) of O_1, can therefore be described by the following equation.

$$R = ([D_t]/K_{o1}) \cdot K_i^2/(K_i + [I])^2 + ([D_t]/K_{o2}) \cdot 2K_i \cdot [I]/(K_i + [I])^2 + ([D_t]/K_{o3}) \cdot [I]^2/(K_i + [I])^2, \quad \mathbf{1}$$

where K_{oX} ($X = 1$ for the complex D–O_1, $X = 2$ for the complex DI–O_1 and $X = 3$ for the complex DI_2–O_1) is

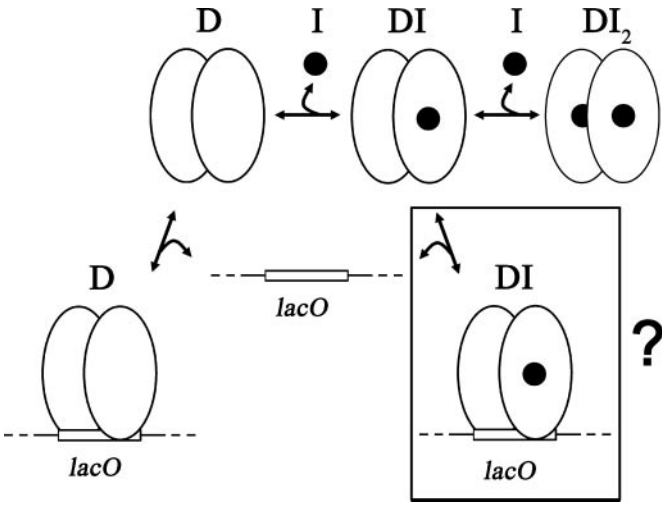

Figure 3. Equilibria of inducer and operator binding of Lac repressor dimers. While it is known that free repressor (*D*) binds strongly to *lac* operator, binding of the complex of the repressor dimer and two molecules of inducer (DI_2) is too weak to play a role at low inducer concentrations. Operator binding of the complex of a Lac repressor dimer with one molecule of inducer (*DI*) cannot be directly measured and is therefore boxed and labelled with a question mark. Closed circles symbolize inducer, open ovals monomers of Lac repressor and the open box *lac* operator (*lacO*).

the equilibrium dissociation constant of the respective *lac* operator–Lac repressor complexes and K_i is the repressor monomer–inducer equilibrium dissociation constant. Figure 3 gives a schematic overview of the relevant equilibria.

The contribution of species DI_2 to repression is negligible at low experimental concentrations of inducer. The operator affinity of the Lac repressor dimer saturated with IPTG (DI_2) is ~1000-fold lower than that of Lac repressor in the absence of inducer (27). For low concentrations of inducer, Equation 1 consequently reduces to the following equation.

$$R = ([D_t]/K_{o1}) \cdot K_i^2/(K_i + [I])^2 + ([D_t]/K_{o2}) \cdot 2K_i \cdot [I]/(K_i + [I])^2. \quad \mathbf{2}$$

Depending on the functional stoichiometry of induction, this equation simplifies further in one of two ways. Model 1: if the functional inducer–repressor stoichiometry of induction is 1:1, only *D* contributes to repression and *DI* is as inefficient in operator binding as is DI_2. In this case, Equation 2 simplifies further to the following equation.

$$R = ([D_t]/K_o) \cdot K_i^2/(K_i + [I])^2. \quad \mathbf{3}$$

Model 2: if the functional stoichiometry is 2:1, only DI_2 loses affinity to operator DNA, and *D* and *DI* have the same high affinity for operator ($K_{o1} = K_{o2} = K_o$). This is the current view (19). In that case, Equation 2 simplifies to the following equation.

$$R = ([D_t]/K_o) \cdot ({K_i}^2 + 2K_i \cdot [I])/(K_i + [I])^2. \quad \mathbf{4}$$

Using these two equations, we performed non-linear regression on the non-cooperative *in vitro* binding data and the two sets of induction data without DNA loops at low inducer concentrations. Taking the unexplained variance as a measure of the fit, we find that model 1 describes the data substantially better. In all three cases, the unexplained variance for model 2 is at least twice that for model 1 (*in vitro*, wt repressor: 1.2% versus 0.6%; *in vivo*, wt repressor: 0.7% versus 0.01%; *in vivo*, dimeric repressor: 1.9% versus 0.7%).

Linear transformation of the alternative Equations 3 and 4 demonstrates that this difference is due to the fact that the best fit of model 2 exhibits a systematic deviation from the data (Figure 4D–F), while the data are well approximated by model 1 (Figure 4A–C). Model 2 is apparently not an appropriate description of the induction data. Both *in vivo* and *in vitro* data can thus be explained assuming a functional inducer–repressor dimer stoichiometry of 1:1. Most of the operator affinity of the Lac repressor dimer is lost upon binding to one molecule of inducer. Operator binding by *DI* can be neglected at low inducer concentrations and at the resolution the induction analysis has.

Table 1 gives the respective inducer and operator binding equilibrium constants of Lac repressor, as determined by non-linear regression analysis using model 1.

DISCUSSION

We demonstrate that the sigmoidality of the induction curve of the wt *lac* operon *in vivo* is a consequence of the relief of cooperative repression through DNA loops during induction. In the absence of DNA loops *in vivo*, induction curves are hyperbolic. There are mainly two reasons why cooperative operator binding (and therefore sigmoidality of induction curves) by Lac repressor had not been seen previously *in vitro*. Firstly, DNA loops are not detectable by filter binding, the most commonly used assay for Lac repressor–DNA interactions for many years (35). Secondly, while DNA loops can be shown with gel-shift assays, they do not form readily on linear *lac* operon DNA as template (41,42).

We therefore used a synthetic construct, carrying two suitably spaced *lac* operators (16 helical turns, assuming a helix repeat of 10.5) with high affinity to Lac repressor (O_1 and O_{id}) for *in vitro* measurements of Lac repressor binding to a DNA template in a loop complex as a function of inducer concentration. While, as expected for a linear template *in vitro*, less pronounced than *in vivo*, the resulting curve is clearly sigmoidal. This confirms our conclusion that sigmoidality of induction of the wt *lac* operon is the consequence of DNA loop formation. Abolishing DNA loops *in vivo* converts a sigmoidal induction curve into a hyperbolic one, and introducing a DNA loop *in vitro* converts a hyperbolic induction curve into a sigmoidal one.

The reconciliation of *in vivo* and *in vitro* observations reopens at the same time the question of functional stoichiometry of Lac repressor–inducer interaction, which had been answered using *in vivo* induction data of the wt lac promoter.

It is a commonly held belief that a plot of log $\{[r - r(0)]/r(0)\}$, where r is $[O_f]/[O_{oc}]$ and $r(0)$ is r at [Inducer] = 0, against log [Inducer] yields the molecular functional induction stoichiometry of inducible systems as the slope of the resulting straight line (13). A slope of ~2 has been reported before for the wt *lac* operon (11). Using this plot on our *in vivo* data on the wt *lac* system, we find a slope of 1.98 (±0.06), consistent

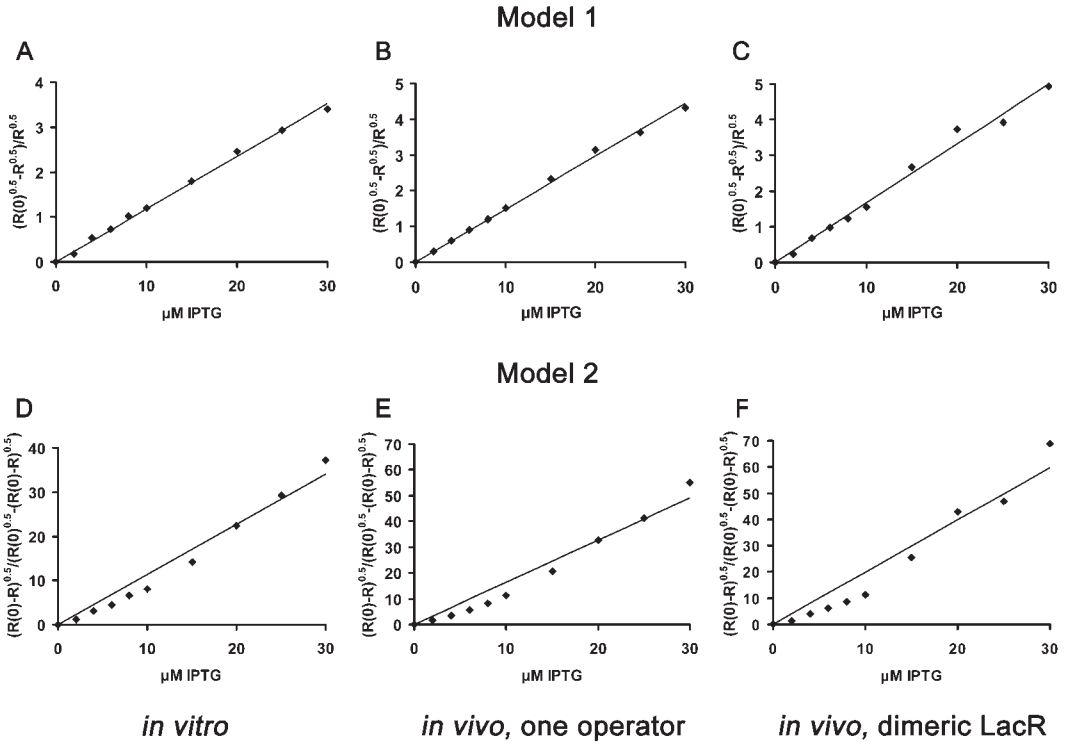

Figure 4. The functional inducer-binding stoichiometry of Lac repressor. Substituting $[D_t]/K_o$ with $R(0)$, the experimental data are transformed using linearizing rearrangements of Equation 3 (Model 1: $(R(0)^{0.5} - R^{0.5})/R^{0.5} = K_i^{-1}[I]$) and 4 (Model 2: $(R(0) - R)^{0.5}/(R(0)^{0.5} - (R(0) - R)^{0.5}) = K_i^{-1}[I]$). Best fit straight lines were derived by linear regression. All plots are of non-cooperative systems. (**A**) *In vitro*, model 1. (**B**) *In vivo*, model 1. The *lac* promoter is controlled by wt tetrameric Lac repressor and the first *lac* operator only. (**C**) *In vivo*, model 1. The *lac* promoter is controlled by dimeric active Lac repressor and all three *lac* operators. (**D**) *In vitro*, model 2. (**E**) *In vivo*, model 2. The *lac* promoter is controlled by wt tetrameric Lac repressor and the first *lac* operator. (**F**) *In vivo*, model 2. The *lac* promoter is controlled by dimeric active Lac repressor and all three *lac* operators.

Table 1. Equilibrium dissociation constants (±SE) for operator binding (K_o) and inducer (IPTG) binding (K_i) of wt tetrameric Lac repressor and the dimeric active mutant 331Stop

	K_o (M)	K_i (M)
In vitro (O_{id})/tet. LacR	$1.26\ (\pm 0.05) \times 10^{-11}$	$8.2\ (\pm 0.4) \times 10^{-6}$
In vivo (O_1)/tet. LacR	$4.39\ (\pm 0.02) \times 10^{-10}$	$6.7\ (\pm 0.1) \times 10^{-6}$
In vivo (O_1)/dim. LacR	$2.44\ (\pm 0.06) \times 10^{-10}$	$6.4\ (\pm 0.4) \times 10^{-6}$

For *in vivo* operator binding, a Lac repressor dimer concentration of 1×10^{-7} M is assumed (24). *In vivo* data are for operator O_1 and *in vitro* data are for the ideal symmetric *lac* operator O_{id} (31,32).

with the previous findings. Our experiments show, however, that this apparent stoichiometry of 2 is coincidental. It is a consequence of cooperative repression through DNA loop formation. For the non-cooperative *lac* systems, *in vivo* and *in vitro*, the slope of this plot is ~1.4–1.5 and thus not compatible with simple stoichiometry, reflecting the simplification of setting $D_t = D + DI_n$ (where operator affinity is lost in the transition from DI_{n-1} to DI_n). We therefore avoided this approximation here.

Our analysis of *lac* induction in the absence of DNA loops is compatible with the suggestion that most of the operator binding of a Lac repressor dimer is lost upon binding of one molecule of inducer. This finding is consistent with what is structurally known about Lac repressor. Operator binding should be weakened as soon as the headpieces of a repressor dimer are out of alignment. This is already the case when one repressor monomer in a dimer undergoes inducer caused allosteric change.

The subsequent binding of Lac repressor dimers to an additional molecule of inducer contributes to induction of the *lac* operon by shifting the equilibrium towards the inactivated form of the repressor dimer (Figure 3).

One could imagine a repressor dimer that exhibits strong negative inducer-binding cooperativity, upon inducer binding of one monomer essentially precluding the second monomer from binding to inducer. Binding to inducer is here described by the following equation.

$$[D] = [D_t] \cdot 0.5K_i/(0.5K_i + [I]). \quad \textbf{5}$$

It is obvious that inactivation of this hypothetical repressor would lag considerably behind that of wt Lac repressor (Figure 5). Thus, the formation of DI_2 has an important thermodynamical role that leads to inactivation of more Lac repressor at lower concentrations of inducer.

The analysis of *lac* induction in the absence of DNA loops yields estimates for *in vitro* and *in vivo* inducer and operator binding constants of Lac repressor, which are comparable with affinities reported in the literature (14,20,29,31,32,43–45).

Interestingly, analysis of inactivation of the *E.coli* Cyt repressor, which is a member of the LacI family of bacterial repressors (46), reveals similarities of the induction process (47). Here, specific binding of the dimeric repressor protein to its operator depends on protein–protein contacts of the repressor monomers to one molecule each of the CAP proteins,

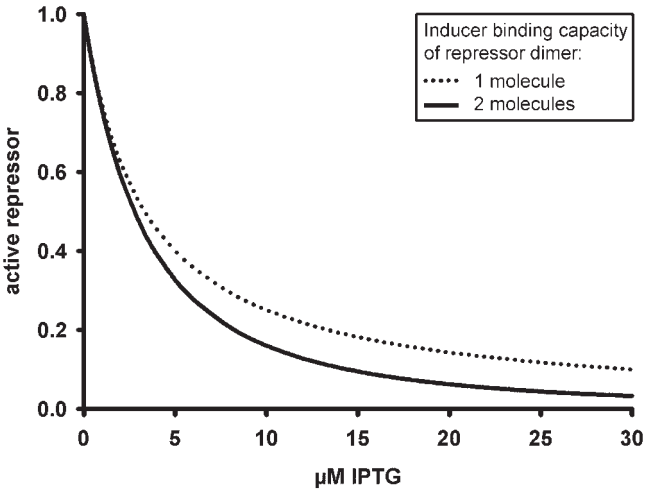

Figure 5. Induction as a function of the inducer-binding capacity of a Lac repressor dimer. Two calculated curves using K_i and K_o from Table 1 (*in vivo*, tet. LacR) are given. Dotted line, inactivation of a (hypothetic) repressor dimer that can only bind to one molecule of inducer (Equation 5). Solid line, inactivation of wt Lac repressor which binds to two molecules of inducer (Equation 3).

which are bound to two CAP binding sites flanking the *cyt* operator. In this system, binding of inducer (cytidine) to the repressor does not directly affect its affinity to operator. Instead, the protein–protein contacts to the CAP proteins are weakened, which then leads to dissociation of the repressor–operator complex. Despite these important differences, also here, binding of one monomer of the repressor dimer to inducer (and, consequently, loss of contact to only one of the two CAP molecules) appears to be sufficient for efficient induction.

It seems not unreasonable to assume that the other members of the family of Lac repressor related proteins, as, for example, the Gal repressor (48), exhibit equivalent mechanisms of induction as the Lac repressor. Future analyses are required to show if this assumption is true.

ACKNOWLEDGEMENTS

We thank Regina Alex and Alexandros Kiupakis for helpful discussions. This work was supported by a grant of Deutsche Forschungsgemeinschaft to B.M.H. The Open Access publication charges for this article were waived by Oxford University Press.

Conflict of interest statement. None declared.

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