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Ligand interactions with lactose repressor protein and the repressor-operator complex: The effects of ionization and oligomerization on binding

Corey J. Wilson [a,b,1], Hongli Zhan [a,c,1], Liskin Swint-Kruse [c], Kathleen S. Matthews [a,b,*]

[a] *Department of Biochemistry and Cell Biology, United States*

[b] *Keck Center for Structural and Computational Biology, Rice University, 6100 Main Street, Houston, TX 77005, United States*

[c] *Department of Biochemistry and Molecular Biology, MS 3030, The University of Kansas Medical Center, Kansas City, KS 66160, United States*

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One of a vanishing breed of gentlemen-scholars, Julian Sturtevant opened his laboratory, his heart, and his mind to innumerable colleagues and their students. An adventurer in life and in science, Professor Sturtevant shared his sense of excitement and anticipation with those who would share the journey. He and his wife Elizabeth made visitors to his laboratory welcome in their home, creating an experience of both scientific and personal expansion for multiple generations of scientists seeking to understand and utilize calorimetric and thermodynamic approaches with biological systems. Once identified as a "national treasure," Julian Sturtevant brought inimitable scientific prowess and personal élan — and his presence is deeply missed. We are honored to be part of this issue dedicated to his memory

Abstract

Specific interactions between proteins and ligands that modify their functions are crucial in biology. Here, we examine sugars that bind the lactose repressor protein (LacI) and modify repressor affinity for operator DNA using isothermal titration calorimetry and equilibrium DNA binding experiments. High affinity binding of the commonly-used inducer isopropyl-β,D-thiogalactoside is strongly driven by enthalpic forces, whereas inducer 2-phenylethyl-β,D-galactoside has weaker affinity with low enthalpic contributions. Perturbing the dimer interface with either pH or oligomeric state shows that weak inducer binding is sensitive to changes in this distant region. Effects of the neutral compound o-nitrophenyl-β,D-galactoside are sensitive to oligomerization, and at elevated pH this compound converts to an anti-inducer ligand with slightly enhanced enthalpic contributions to the binding energy. Anti-inducer o-nitrophenyl-β,D-fucoside exhibits slightly enhanced affinity and increased enthalpic contributions at elevated pH. Collectively, these results both demonstrate the range of energetic consequences that occur with LacI binding to structurally-similar ligands and expand our insight into the link between effector binding and structural changes at the subunit interface.

Keywords: Lactose repressor; Isothermal titration calorimetry; Binding affinity; Allosteric behavior; Inducer; Anti-inducer

1. Introduction

Understanding and predicting the energetic bases for selecting one compound from a mixture of molecules are central problems in contemporary biophysics. Knowledge of which features contribute to specificity and precision in protein–protein, protein–ligand, and protein–DNA interactions is required to design new materials with prescribed properties. Generally, high-resolution structures of biomolecular complexes demonstrate complementarity of interacting surfaces and show the requisite orientations of interacting groups that provide high affinity interactions. However, the strength of these interactions (e.g., hydrogen bonding, salt bridges, and the hydrophobic effect) is best determined from energetic measurements. In addition, these measurements provide information about functional consequences of binding, such as when binding one ligand allosterically affects binding of a second.

* Corresponding author. Department of Biochemistry & Cell Biology, Rice University, 6100 Main Street, Houston, TX 77005, United States. Tel.: +1 713 348 6149; fax: +1 713 348 4871.
E-mail address: ksm@bioc.rice.edu (K.S. Matthews).
[1] These two authors contributed equally to this work.

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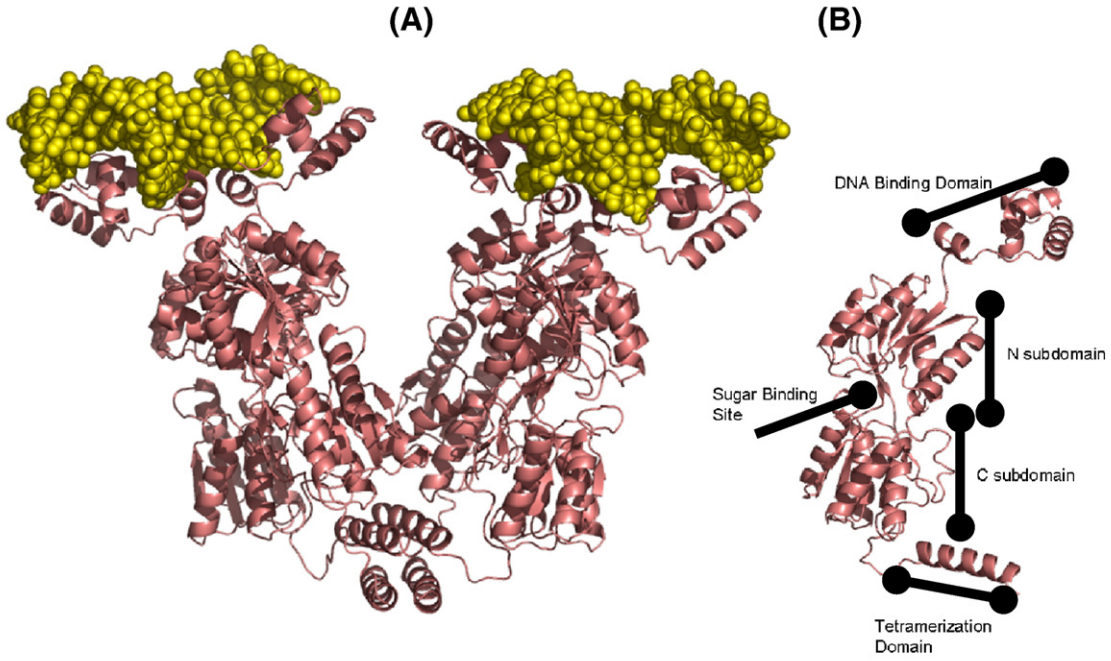

Fig. 1. LacI structure. (A) Structure of LacI tetramer bound to operator DNA. The coordinates are from Protein Data Bank file 1lbg [6]. The operator DNA is represented by space filled structure. (B) Structure of the LacI monomer with sub-regions labeled: N-terminal DNA binding domain (residues ~1–60), core domain (residues ~60–340) that comprises the N- and C-subdomains, and tetramerization domain (residues ~340–360). The sugar binding site is located between the N- and C-subdomains. Note that this site is structurally remote from the N-terminal DNA binding domain, despite the impact of sugar binding on DNA binding affinity.

The lactose repressor protein (LacI)[2] has served as a paradigm for studies of gene regulation, ligand recognition, and allosteric response [1–3]. LacI is a tetramer comprised of identical ~37.5 kDa monomers (Fig. 1A) [3–6]. Monomers are separated structurally into three domains: (1) a DNA binding domain (residues 1–50) and hinge region (residues 50–60) that are engaged in operator DNA recognition, (2) a core domain (residues 60–340) that binds sugar ligands and encompasses the monomer–monomer interface, and (3) a C-terminal domain (residues 340–360) that comprises the tetramerization domain (see Fig. 1B) [6,7]. The binding functions of the two domains are allosterically linked. That is, the affinity of the DNA-binding domains for operator is altered when the LacI core domain binds specific sugar ligands, and *vice versa*. However, a variety of sugar derivatives can bind in this same pocket with distinct effects on DNA-binding [8]: Inducers diminish DNA-affinity, neutral sugars have no effect, and anti-inducers strengthen affinity for DNA. Interestingly, however, the sugar effector ligands in the crystallographic protein structures are not well-resolved [6,7,9].

[2] LacI regulates transcription *via* negative control of the *lac* operon structural genes (β-galactosidase, lactose permease and thiogalactoside transacetylase) that are responsible for transport and metabolism of lactose in *E. coli*. In the absence of glucose, *E. coli* can use lactose as a carbon source. Under these conditions, basal levels of lactose permease and β-galactosidase transport and convert a portion of lactose into 1,6-allolactose, which binds to LacI and induces a conformational change. This structural shift reduces LacI affinity for the largely palindromic operator sequence, located between the promoter and structural genes, and allows RNA polymerase to transcribe the structural genes [1–3].

Since the seminal study by Barkley and colleagues [8] that used equilibrium dialysis to characterize 29 sugar derivatives, the majority of LacI studies (and biotechnological applications) have utilized the gratuitous inducer isopropyl-β,D-thiogalactoside (IPTG). IPTG binds to LacI with high affinity [8], its binding isotherms can be determined by fluorescence spectroscopy [10], previous measurements have explored its enthalpy of binding [11], and the compound has become widely available. However, understanding LacI as a paradigm for allosteric regulation necessitates our examining functional effects that result from binding a wider range of allosteric ligands to the sugar-binding site. Many of these molecules contain aromatic residues that interfere with fluorescence spectroscopic analysis [8,10], and very few are available with radiolabels; thus, direct measurements by equilibrium dialysis are not possible. Although changes in circular dichroism have been used to examine sugar binding [12], this method may not easily adapt for a range of ligands. Further, neither competition with radiolabeled-IPTG in equilibrium dialysis nor circular dichroism measurements are practical for routinely determining binding parameters for LacI allosteric variants. Finally, none of these methods provide thermodynamic information beyond binding affinity (which yields ΔG_{obs}). Therefore, we have utilized the advancements offered by isothermal titration calorimetry (ITC) [11,13] to detail the thermodynamic parameters for a subset of LacI sugar ligands: inducers IPTG and 2-phenylethyl-β,D-galactoside (PhEG), neutral sugar o-nitrophenyl-β,D-galactoside (ONPG), and anti-inducer o-nitrophenyl-β,D-fucoside (ONPF) [8,12,14,15].

In this study, we also explored functional consequences of LacI binding to various effector ligands using other thermodynamic approaches to monitor the allosteric impact on repressor–operator binding affinity. We varied both pH and oligomeric state to perturb the monomer–monomer interface, which is known to change significantly upon effector binding [6,9,15]. In comparison to measurements made at neutral pH, LacI binding affinity for inducer IPTG was previously shown to be diminished and cooperative in Tris–HCl buffers at elevated pH or in the presence of DNA [16–20]. However, elevated pH has no significant effect on operator DNA binding [8,21,22]. To vary oligomerization, we use the −11 variant, which is not capable of forming tetramers [19]. At the low protein concentrations required to measure DNA binding, this protein is in equilibrium between dimeric and monomeric species [19,23,24]. Altering either oligomeric state or pH results in intriguing differences for LacI binding to the four different ligands used in this study.

2. Experimental

2.1. Protein expression and purification

Experiments utilized wild-type LacI tetramer (which does not show detectable dissociation at even 10^{-13} M protein) and the "−11" variant, which cannot form tetramer and at very low concentrations is in equilibrium between monomer and dimer ($K_d \sim 8\times10^{-8}$ M in the absence of effector) [23]. Proteins were expressed as described previously [18,19] in BL26 blue cells (Novagen) that were cured of the episome carrying the I^q promoter and the *lac*I gene [25], under standard conditions (2xYT rich liquid media supplemented with 50 μg/ml ampicillin, at 37 °C, incubated for ~20 h). Bacteria were harvested *via* centrifugation, and the cell pellet was resuspended in breaking buffer (0.2 M Tris–HCl, pH 7.6, 200 mM KCl, 0.01 M $Mg(OAc)_2$, 0.3 mM dithiothreitol, 5% glucose) and supplemented with lysozyme (3 mg/l), PMSF (~0.5 mg/l), and DTT (0.3 mM) before freezing (−20 °C). Purification of LacI was initiated by thawing the cell pellet on ice, then DNaseI (~3 mg/ml) was added to digest genomic DNA. Post-centrifugation, the crude supernatant was subjected to 37% ammonium sulfate precipitation. The ammonium sulfate pellet was resuspended in 0.09 M KP buffer (0.09 M potassium phosphate, 5% glucose, 0.3 mM DTT, pH 7.5) and dialyzed against the same buffer. After centrifugation to remove any precipitate, the supernatant was loaded onto a phosphocellulose column equilibrated in 0.09 M KP buffer. Following washing with 0.12 M KP buffer, the LacI tetramer was eluted with a gradient from 0.12 to 0.30 M KP buffer. For the LacI/−11 dimer, the process was similar, but dialysis was against 0.05 M KP buffer, and the protein was eluted from the phosphocellulose column in 0.12 M KP buffer. Protein concentrations were determined by absorbance at 280 nm using the extinction coefficient for wild-type *lac* repressor ($\varepsilon_{280}=0.6$ ml $mg^{-1}\cdot cm^{-1}$). Purified LacI was 95–99% pure according to SDS-PAGE.

2.2. Buffer/sample preparation

To avoid potential problems that may arise during ITC from components in regular KP buffer (e.g., DTT and glucose), all experiments reported herein were performed in 50 mM phosphate buffer, with pH at either 7.5 or 9.2. Buffers were made by either adjusting the pH of 50 mM K_2HPO_4 to 7.5 using 50 mM KH_2PO_4 or adjusting the pH of 50 mM K_2HPO_4 to 9.2 using 50 mM K_3PO_4. Protein samples in column elution buffer were dialyzed against one of these two buffers for ~1 h each for six buffer exchanges. Buffer to sample ratio was ≥100. To insure the completion of dialysis and sample quality, pH of the protein samples was monitored in addition to diagnostic ITC binding to IPTG.

2.3. Far/near-UV circular dichroism

To assess the asymmetric environment of the aromatic residues for LacI and the global secondary structure in 50 mM potassium phosphate compared to those previously determined in 50 mM Tris–HCl (pH at 7.5 or 9.2), the near- and far-UV CD spectra were recorded between 240–320 and 190–250 nm, respectively. Circular dichroism spectra were recorded on an Aviv 62A DS spectropolarimeter calibrated with an aqueous solution of *d*-10-(+)-camphorsulfonic acid. Data were collected in 1.0–0.5 nm intervals with an integration time of 5 s. Measurements were performed using a 10 mm quartz cell with the temperature maintained at 25 °C. The CD signals from the cuvette and sample lacking the protein were subtracted from all recorded spectra. The ellipticity is reported as the mean residue molar ellipticity ([θ], deg cm^2 $dmol^{-1}$) calculated from:

$$[\theta] = [\theta]_{obs}(mrw)/10cl \quad (1)$$

where $[\theta]_{obs}$ is the ellipticity measured in degrees, (mrw) is the mean residue molecular weight, *c* is the protein concentration in $dmol/cm^3$, and *l* is the optical path length of the cell (in centimeters).

2.4. Isothermal titration calorimetry

Inducer binding to LacI and LacI/−11 in 50 mM potassium phosphate buffer, pH 7.5 and 9.2, was investigated by isothermal titration calorimetry (VP-ITC, MicroCal) at 25 °C. Protein and ligand samples were filtered through 0.22 μm nitrocellulose membranes and degassed at 25 °C (ThermoVac, MicroCal) before loading into the ITC cell and syringe. Prior to ligand–protein binding experiments, reference titrations (injecting ligand into 50 mM potassium phosphate buffer, pH 7.5 and 9.2) were carried out. A typical injection schedule included the addition of 5–10 μl samples of ligand to 30 μM protein monomer (LacI and LacI/−11; 1.4 ml volume) with 20–30 injections at 3 to 5 min intervals. Concentrations of ligand utilized were 1–1.5 mM IPTG and 7.5–15 mM for the remaining sugars. The C-values used in this study were selected to establish a clear post-transition baseline while maximizing the number of pre-transition/transition points; intermediate

(unsaturated) reaction setups resulted in an incomplete post-transition and sloped pre-transition. To ensure that the concentration regimes selected for a given ITC experiment did not lead to early saturation of the protein, several titrations were conducted using a range of ligand concentrations. In all cases, the pre-saturating baseline condition resulted in a sloped pre-transition. The ligand-to-buffer reference titration was subtracted before the resulting binding isotherms were fit to a single binding-site model using Marquardt non-linear least-square analysis (Origin 5.0).

2.5. DNA binding/operator release

DNA binding and operator release experiments were performed using filter binding assays with the natural operator sequence, O^1 (TGTTGTGTGGAATTGTGAGCGGATAA-CAATTTCACACAGG; Biosource International; Camarillo, CA) [18,26–29]. The top and bottom strands were hybridized in polynucleotide kinase buffer (70 mM Tris–HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 7.6). The DNA was then labeled with [^{32}P]-ATP using polynucleotide kinase and purified using a Nick column (Amersham Biosciences, Uppsala, Sweden) to remove unincorporated nucleotide. In binding assays, operator concentration was set at least 10-fold below the K_d ($\sim 1.5\times10^{-12}$ M), whereas protein concentration was varied. After filtration through nitrocellulose paper (Schleicher and Schuell, Keene, NH), the retained radioactivity was detected and quantified by a Fuji phosphorimager. If present, the sugar concentrations were set as: 2.2 or 3 mM o-nitrophenyl-β,D-fucopyranoside (ONPF) (Sigma, St. Louis, MO), 1.5 or 2 mM o-nitrophenyl-β,D-galactopyranoside (ONPG) (Sigma, St. Louis, MO), 5 mM (pH 7.5) or 10 mM (pH 9.2) phenylethyl-β,D-galactopyranoside (PhEG) (Sigma, St. Louis, MO), and 1 mM IPTG (RPI, Troy, NY). Data were analyzed with IgorPro (Wavemetrics, CA) to determine the DNA binding affinities, using the following equation:

$$Y_{obs} = \left(Y_{max} * \frac{[LacI]^n}{K_d^n + [LacI]^n}\right) + c \quad (2)$$

Here, Y_{obs} is the observed level of retained radioactivity, Y_{max} is maximal level of retained radioactivity, K_d is the equilibrium dissociation constant, c is the background radioactivity without the formation of protein–DNA complex, and n is the Hill coefficient, which is generally ~ 1 for LacI; however, this value may be >1 for LacI/−11 due to thermodynamic coupling between assembly and DNA binding [23].

To measure the effect of sugars on DNA binding – termed operator release and capture – DNA concentration was 1.5×10^{-12} M, and protein concentration was set to attain $\sim 80\%$ saturation of DNA (see figure legend for precise concentrations). The protein was allowed to incubate with DNA for ~ 30 min before sugars (range was varied from 10^{-8} to 10^{-2} M dependent on individual sugars) were added to release (PhEG and IPTG) or capture (ONPF and ONPG at pH 9.2) operator DNA. After filtration through nitrocellulose filter paper, retained radioactivity was quantified using a Fuji phosphorimager. The data from capture experiments were fit with a modified version of Eq. (2), in which the parameter K_d was substituted with $[sugar]_{mid}$ and [LacI] was replaced with [sugar]. Data from operator release experiments were fit using:

$$Y_{corr} = Y_{max} - \left(Y_{max} * \frac{[sugar]^n}{[sugar]_{mid} + [sugar]^n}\right) + c \quad (3)$$

this equation is derived from Eq. (2) to accommodate decreasing signal as a function of sugar binding for IPTG and

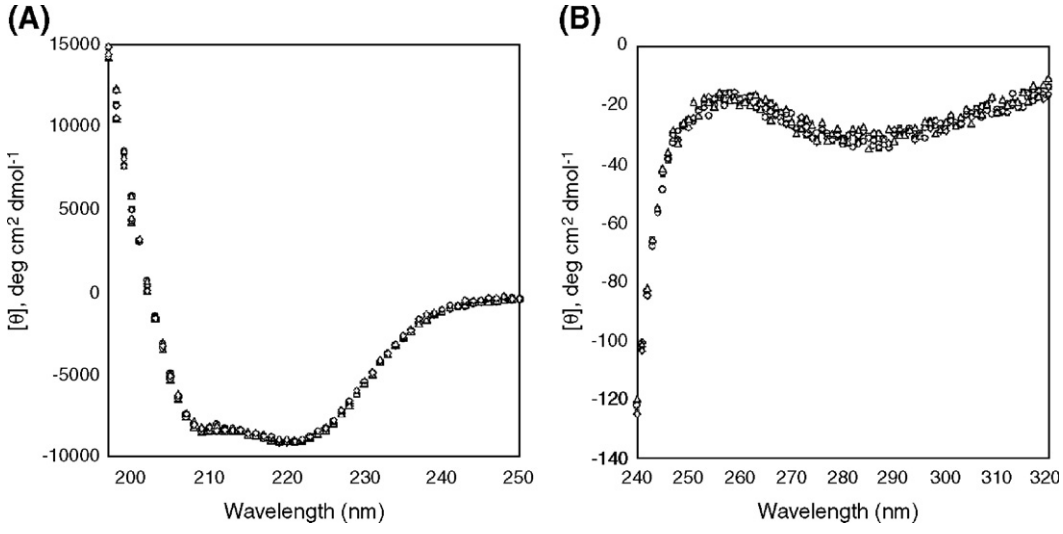

Fig. 2. Circular dichroism spectra. Measurements were made as described in Experimental. For both panels: LacI, 50 mM phosphate buffer, pH 7.5 (diamonds) and pH 9.2 (circles). LacI, 50 mM Tris–HCl buffer, pH 7.5 (triangles) and pH 9.2 (squares). (A) Far-UV circular dichroism spectra report on secondary structure content and are nearly identical under all conditions of buffer and pH for both wild-type tetramer (shown in the figure) and dimeric LacI/−11 protein. Binding to IPTG does not alter the spectral features (data not shown). (B) The near-UV CD bands between 240 and 320 nm of LacI at both pHs report similar features that are unchanged by IPTG presence (data not shown in the presence of IPTG). This similarity suggests that no changes are observed in the asymmetric environment around the aromatic residues, especially tryptophan.

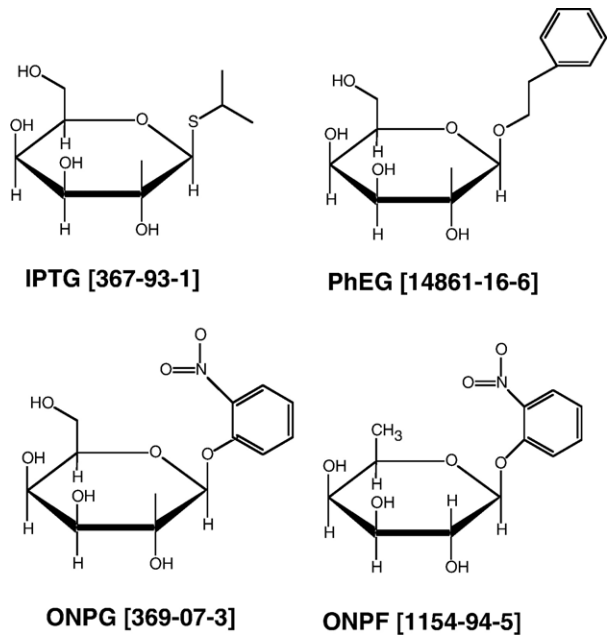

Fig. 3. Structure of sugar ligands. The sugars employed in this study are the inducer isopropyl-β,D-thiogalactopyranoside (IPTG), the anti-inducer o-nitrophenyl-β,D-fucopyranoside (ONPF), the neutral compound o-nitrophenyl-β,D-galactopyranoside (ONPG), and the weak inducer 2-phenylethyl-β,D-galactopyranoside (PhEG). The CAS Registry numbers for each compound are indicated.

PhEG. Y_{obs} is measured radioactivity at a specific sugar concentration; Y_{max} is the maximal change in radioactivity that results from binding of saturating sugar; n is the Hill coefficient; $[sugar]_{mid}$ is the concentration of sugar where 50% operator is released or captured; and c is the background value.

3. Results

3.1. Global binding context and repressor structure

Traditionally, thermodynamic characterization of LacI has been carried out in Tris–HCl buffers, so that ionic strength could be easily controlled during studies of DNA binding. Unfortunately, the temperature dependence for ionization of this buffer is not suitable for ITC. Phosphate buffer is optimal for this technique; however, based on attempts to crystallize the protein, previous ITC studies [11], and information from mass spectroscopy, we deduce that phosphate may bind specifically to LacI (R. Shreter, C. Falcon, and K. Matthews, unpublished data), presumably to the DNA-binding domain which has a strong net positive charge. Therefore, we chose to keep the concentration of phosphate ion constant for the two pH conditions, despite the consequence of variations in ionic strength. This approach is justified by previous observations that changes in pH at higher salt concentrations do not significantly affect IPTG binding [11,16].

Further, we ascertained that the overall LacI structure was not changed in phosphate buffers. The far-UV spectra, reporting on secondary structure content, were nearly identical for LacI wild-type and the −11 variant in phosphate and in Tris–HCl buffers, at pH 7.5 and 9.2, free and bound to IPTG. Fig. 2 shows spectra in both buffers and pHs for LacI. All measured spectra comprise two negative Cotton peaks around 208 and 222 nm, reflecting the expected features for α-helical structure.

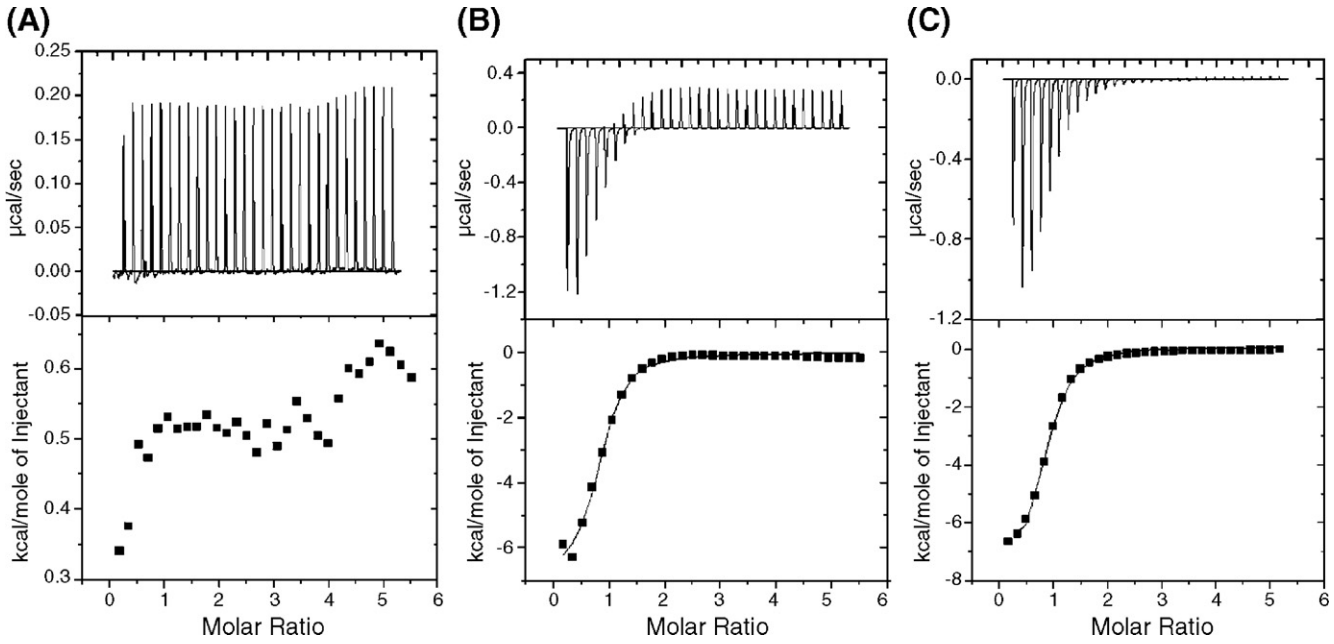

Fig. 4. Glucose effects on isothermal titration calorimetric results. Representative ITC titrations of LacI with IPTG are shown at different time-points during sample preparation. The purification protocol utilizes 5% glucose in the buffer, and we demonstrate that this component must be removed by extensive dialysis. The dialysis time for each exchange was 1 h each, and the volume ratio of dialysate buffer to sample was >100:1. (A) ITC isotherm following two exchanges of external dialysate buffer. (B) ITC isotherm following four exchanges of external dialysate buffer (C) ITC isotherm following six exchanges of external dialysate buffer.

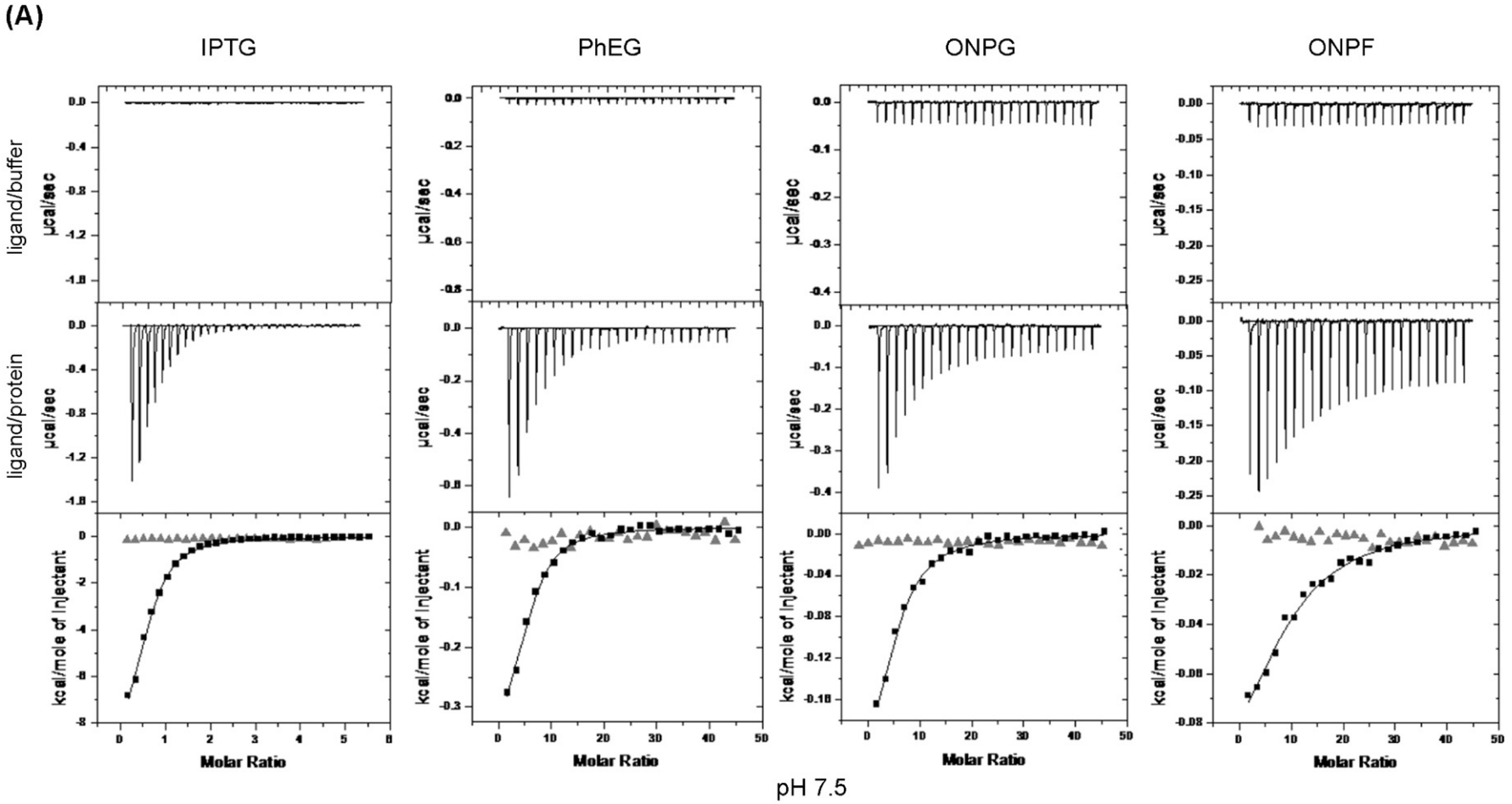

Fig. 5. Isothermal titration calorimetry binding isotherms for LacI. Experiments were carried out as described in Experimental for LacI in 50 mM phosphate buffer at pH 7.5 (A) and 9.2 (B). The ligands examined included IPTG, PhEG, ONPG, and ONPF. Values derived from multiple determinations for LacI and for LacI/−11 are provided in Tables 1 and 2. Results from control titrations with buffer are incorporated in each panel (top section) and are indicated in the binding curves by gray triangles.

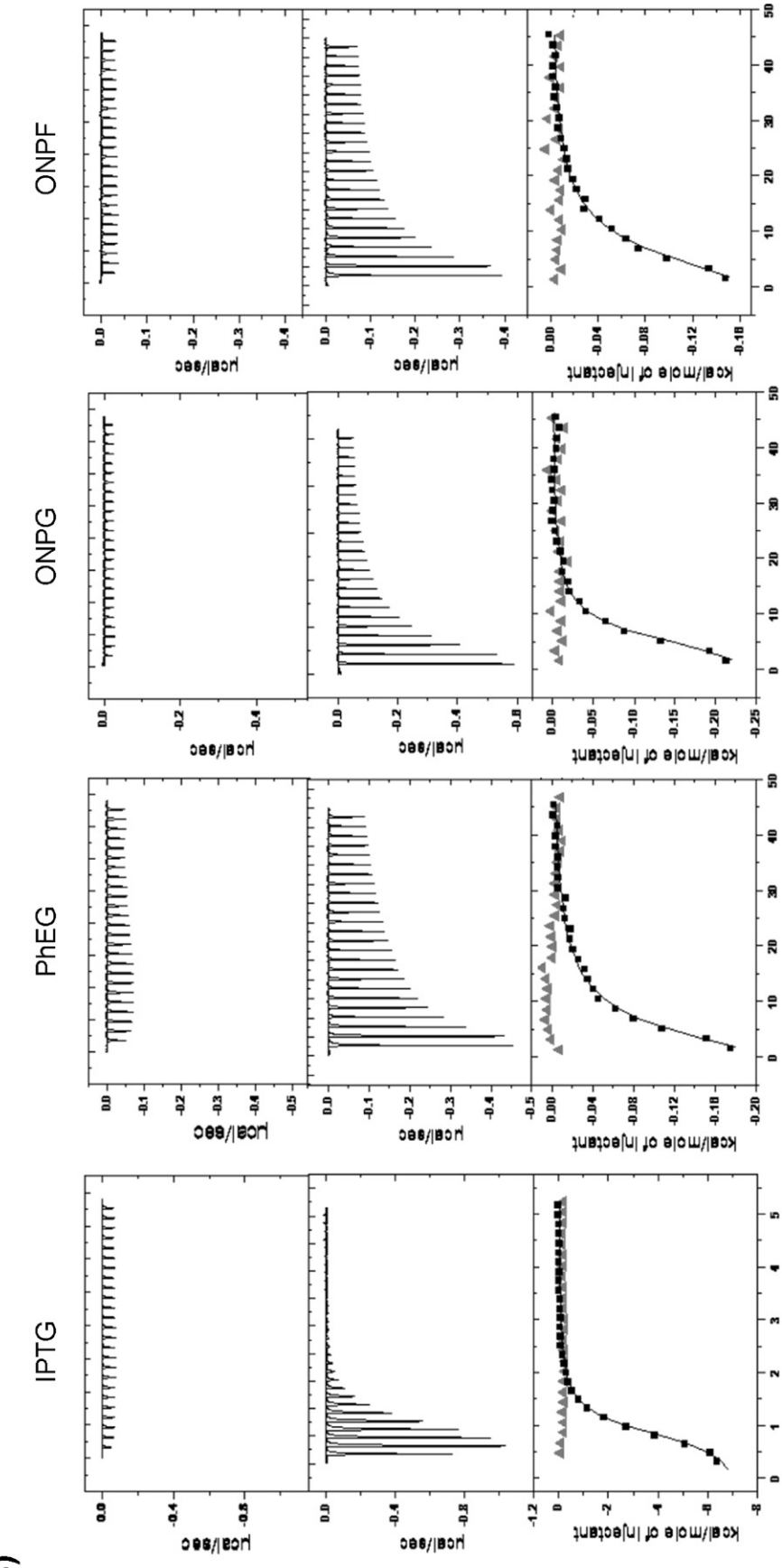

Fig. 5 (*continued*).

Table 1
Isothermal titration calorimetry binding constants[a]

	K_d ($M \times 10^{-6}$)			
	pH 7.5		pH 9.2	
	LacI	LacI/−11	LacI	LacI/−11
IPTG inducer	2.8±0.2	2.2±0.3	4.4±0.2	3.6±0.2
PhEG weak inducer	50±3	56±2	140±10	>500
ONPG neutral	83±4	150±20	56±2	67±5
ONPF anti-inducer	160±20	160±20	100±20	64±2
IPTG[b] fluoresence	4.5±0.3	3.0±0.2	6.6±0.5	4.7±0.5

[a] Values reported represent the mean and standard deviation for at least 3 separate experiments conducted as described in Experimental.
[b] Data for IPTG binding by fluorescence in phosphate buffer are provided for comparison. For further comparison, binding constants from Barkley et al. [8] determined by equilibrium dialysis in 0.1 M potassium phosphate, pH 8.0, 1 mM DTT, 1 mM azide were as follows: IPTG, 0.83×10^{-6} M; ONPF, 140×10^{-6} M; ONPG, 110×10^{-6} M; PhEG, 200×10^{-6} M. Note that in some cases, bovine serum albumin was added to the equilibrium dialysis experiments at 100 μg/ml [8]. IPTG binding by equilibrium dialysis was determined directly with the radiolabeled compound, whereas the remaining sugars were measured by competition with radiolabeled IPTG.

Moreover, the near-UV CD bands, which primarily report the tertiary structure asymmetrically formed around tryptophan residues 201 and 220, are essentially the same for all proteins in all conditions (free and bound). Therefore, local asymmetric tertiary structure surrounding the aromatic residues is largely unperturbed, and the gross structural features are identical under different ligand conditions.

3.2. Isothermal titration calorimetry — rationale and impediments

Fluorometry is the standard method for examining IPTG ligand binding to LacI [10,16,30]; however, this method cannot be utilized with the large subset of LacI effecting ligands that contain aromatic moieties and absorb ultraviolet wavelengths of interest. Although circular dichroism has been used to explore binding [12], isothermal titration calorimetry (ITC) provides the capacity to examine thermodynamic parameters for sugars in each class of effector previously identified – inducer (PhEG), anti-inducer (ONPF) and neutral (ONPG) (Fig. 3) – and compare them to those for traditional gratuitous inducer, IPTG [11].

Glucose is often utilized as a solute in purification of proteins to enhance stabilization [31], and its inclusion in LacI purification buffers ensures maximum activity. However, glucose is also a weak anti-inducer for LacI [8]. Due to this latter activity (most likely mediated through specific binding to the sugar binding pocket), we found that residual glucose impeded analysis of ligand binding by ITC, as demonstrated in Fig. 4. Indeed, the effects of glucose on IPTG binding by ITC can be utilized to assess the effectiveness with which this compound has been removed by dialysis. The competition between glucose and other effector sugars was not detected by previous techniques used to measure binding affinities. These observations emphasize that careful attention to the buffer components is required for studies that utilize ITC for quantitative determination of binding and thermodynamic parameters.

3.3. LacI ligand binding by isothermal titration calorimetry

Binding affinities for sugars from each class of effector to LacI tetramer or the LacI/−11 dimeric variant were compared using ITC at pH 7.2 and 9.5 (Fig. 5, Table 1). Both proteins bind IPTG with approximately the same affinity, independent of pH, and with similar enthalpic contributions. The results at pH 9.2 are similar to those obtained by Donner et al. [11] in 0.5 M KCl, 0.1 M phosphate buffer at elevated pH, but do not correspond to their results in the same buffer at neutral pH, perhaps because of differences in buffer content and/or ionic strength. Values derived from ITC are comparable to those determined by fluorescence for IPTG.

The weak-inducer PhEG binds to LacI with ~3-fold weaker affinity at pH 9.2, whereas LacI/−11 binds with >10-fold weaker affinity at pH 9.2. The enthalpic contributions to binding are small at both pHs (Table 2). In contrast, the neutral sugar ONPG is sensitive to pH changes, binding to both LacI/−11 and LacI with greater affinity and slightly more enthalpic contribution at pH 9.2. The anti-inducer ONPF is also sensitive to altered pH, again with higher affinity and somewhat increased enthalpic contribution at pH 9.2. ITC clearly provides a robust approach for dissecting the impact of variant conditions and protein sequences on binding parameters for the entire range of LacI effectors.

3.4. DNA binding

Operator affinity was determined using nitrocellulose filter binding for LacI/−11 and wild-type tetramer at pH 7.5 and 9.2 in the presence of each ligand examined by ITC (Fig. 6, Table 3). Buffer utilized was the same as that in ITC measurements of effector binding. The apparent operator binding affinity for LacI/−11 protein is diminished, as expected due to the equilibrium between monomeric and dimeric species previously detected for this variant [23]. Note that DNA binding in the absence of effector is minimally influenced by pH. Moreover, the data confirm that effector ligands influence DNA binding affinity for both LacI/−11 and tetrameric LacI. Anti-inducer

Table 2
Isothermal titration calorimetry ΔH_{obs} values[a]

	ΔH_{obs} (kcal mol^{-1})			
	pH 7.5		pH 9.2	
	LacI	LacI/−11	LacI	LacI/−11
IPTG inducer	−7.6±0.1	−8.5±0.2	−7.6±0.2	−7.5±0.5
PhEG weak inducer	−0.40±0.01	−0.15±0.04	−0.42±0.02	ND[b]
ONPG neutral	−0.28±0.03	−0.18±0.04	−0.34±0.01	−0.43±0.03
ONPF anti-inducer	−0.12±0.06	−0.28±0.02	−0.26±0.3	−0.35±0.02

[a] Values for ΔH_{obs} were determined as described in Experimental. Values reported represent the mean and standard deviation for at least 3 separate experiments.
[b] Not detectable.

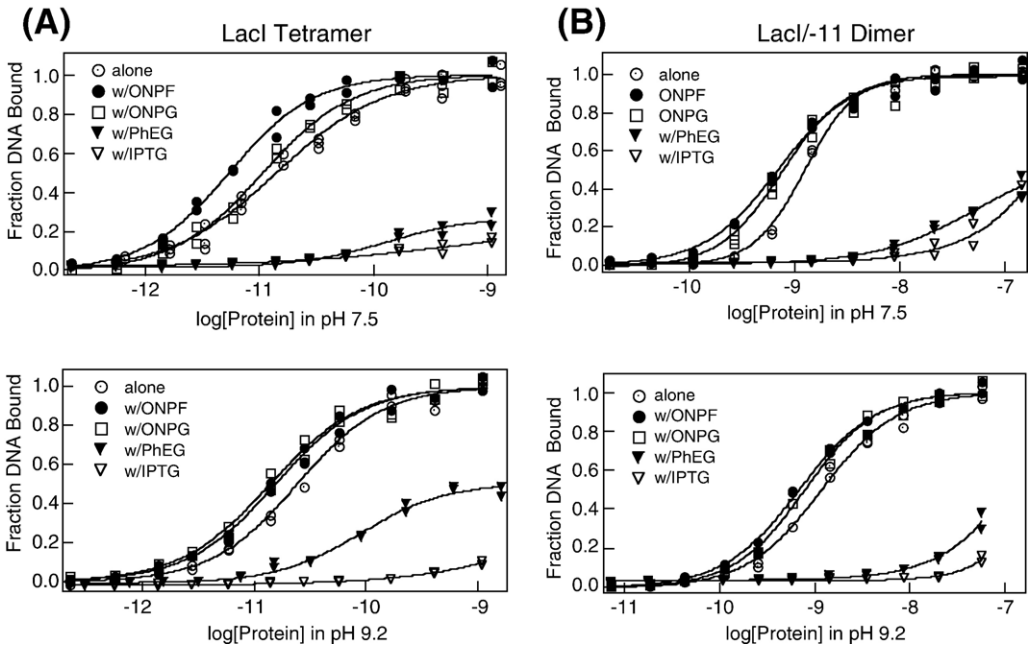

Fig. 6. Operator binding measurements for LacI/−11 and LacI wild-type tetramer at pH 7.5 and 9.2. (A) Wild-type LacI tetramer. (B) LacI/−11 dimer. Experiments were performed in buffer containing either 50 mM potassium phosphate, pH 7.5, or 50 mM potassium phosphate, pH 9.2. The experiments were conducted as described in Experimental with operator concentration at 1.5×10^{-12} M. Sugar concentrations were 2.2 or 3 mM for ONPF, 1.5 or 2 mM for ONPG, 5 or 10 mM for PhEG, and 1 mM for IPTG.

(ONPF) increases DNA-binding affinity of both wild-type and LacI/−11 by about 2-fold under all conditions. The presence of "neutral" ligand (ONPG) does not affect DNA-binding for wild-type LacI at pH 7.5, but increases affinity for DNA at pH 9.2. Surprisingly, this compound converts to an anti-inducer for LacI/−11 at both pHs. Likewise, LacI/−11 is more sensitive to the weak inducer PhEG than is the tetrameric protein: PhEG is a very effective inducer for LacI/−11 at both pH conditions, whereas LacI tetramer is less sensitive to this sugar at pH 9.2. This disparity may derive from differential stability of the LacI/−11 monomer–monomer interface.

3.5. Operator release and capture

Operator release/capture experiments monitor the concentration of ligand required to release radiolabeled DNA from or capture the DNA into a complex with repressor protein. These experiments are very sensitive to altered operator affinities in the presence of various effectors. Using dot blot analysis with a single filter, multiple conditions can be monitored simultaneously. We employed this method for ONPF, ONPG, PhEG, and IPTG; results are presented in Table 4 and Fig. 7.

For LacI in the presence of IPTG at pH 7.5, our data compare very well with values reported in the literature, and the oligomeric state does not appear to have a significant effect on operator release, again consistent with results obtained in Tris–HCl buffer [32,33]. When the repressor is in the presence of the weak inducer PhEG, substantially higher ligand concentrations are required for release, particularly at pH 9.2.

Table 3
Operator binding constants for LacI and LacI/−11[a]

	K_d ($M\times10^{-11}$)			
	pH 7.5		pH 9.2	
	LacI	LacI/−11	LacI	LacI/−11
No ligand	2.1±0.5	110±20	2.0±0.3	120±8
+IPTG	>10000	>10000	>10000	>10000
+PhEG	>20	>10000	9.2±3	>10000
+ONPG	2.0±0.4	72±20	1.2±0.2	73±10
+ONPF	1.2±0.06	62±20	1.3±0.1	70±10

[a] Operator binding experiments were performed in buffer containing 50 mM potassium phosphate with pH at 7.5 or 9.2. Operator O^1 DNA concentration was below 1.5×10^{-12} M for all proteins. Values shown represent an average of 3–5 measurements, each in duplicate or triplicate, with standard deviation.

Table 4
Operator release and capture: sugar-binding properties of LacI[a]

	$[sugar]_{mid}$ ($M\times10^{-6}$)			
	pH 7.5		pH 9.2	
	LacI	LacI/−11	LacI	LacI/−11
IPTG	2.8±0.6	2.0±0.09	5.6±1	3.3±0.5
PhEG	210±40	320±20	1300±200	1800±300
ONPG	NBD[b]	170±40	190±20	180±40
ONPF	190±30	140±20	170±20	130±40

[a] Operator release and capture experiments were performed in buffer containing 50 mM potassium phosphate at pH 7.5 or 9.2. Operator concentration was 1.5×10^{-12} M, and protein concentrations were 3.0×10^{-9} M for LacI/−11 at both pH values; the wild-type LacI concentration was 6×10^{-11} M at pH 7.5 and pH 9.2. Values shown represent an average of 3–4 measurements, each in duplicate or triplicate, with standard deviation.

[b] NBD: no binding detected.

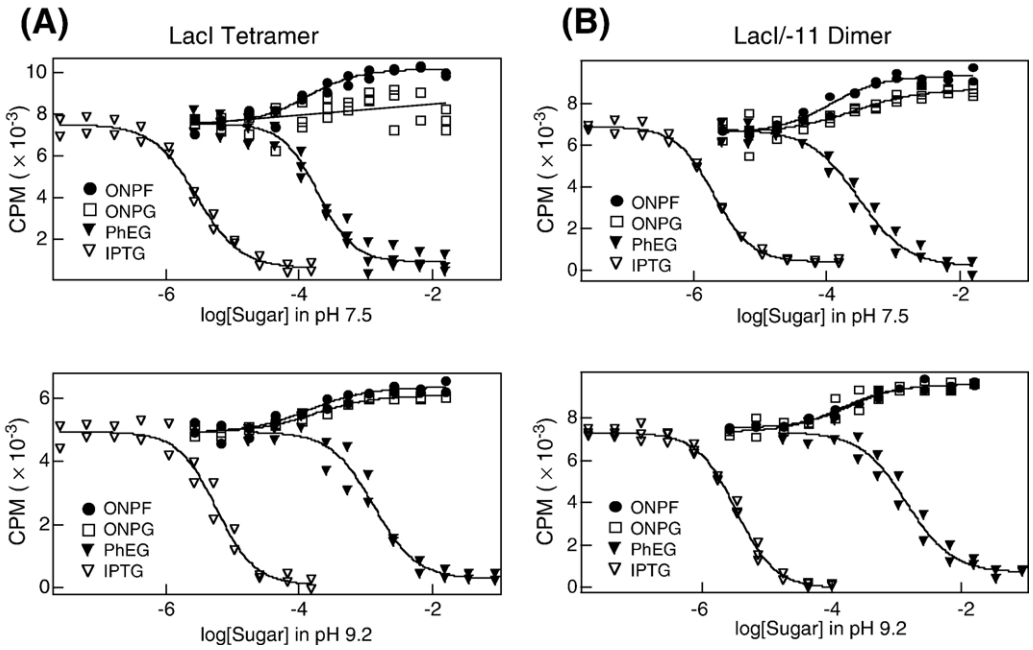

Fig. 7. Operator release and capture. Experiments were conducted as described in Experimental. (A) LacI tetramer at pH 7.5 and 9.2. (B) LacI/−11 dimer at pH 7.5 and 9.2. The buffer was 50 mM potassium phosphate with pH as noted. Sugar concentrations were varied as indicated. Operator concentration was 1.5×10^{-12} M, and protein concentrations were 3.0×10^{-9} M for LacI/−11 at both pH values or 6×10^{-11} M for wild-type LacI at both pH values. Data are shown for a single experiment, and values from multiple experiments are reported in Table 4.

In the presence of the anti-inducer ONPF, LacI and LacI/−11 capture operator DNA at similar ligand concentrations at both pHs examined. Interestingly, the neutral ligand (ONPG) converts to an anti-inducer at pH 9.2, and at both pH values for LacI/−11. Although the binding affinity of LacI for IPTG generally parallels the value of $[\text{sugar}]_{\text{mid}}$ needed to release operator [33], we do not necessarily expect these values to be equivalent to ITC-determined binding affinities for other ligands: (1) Release and capture experiments are performed in the presence of DNA, whereas reported binding affinities are in the absence of DNA. (2) Any other linked thermodynamic equilibria will be encompassed by release/capture experiments, such as equilibria between open/closed LacI conformations or monomer–dimer equilibria. Indeed, for LacI/−11, release/capture experiments are carried out at protein concentrations well below the K_d for the monomer–dimer equilibrium [23], whereas protein concentrations in ITC experiments are well above that value. Nonetheless, the values reported by these two methods for ONPF and ONPG generally parallel. In contrast, the value of $[\text{sugar}]_{\text{mid}}$ for PhEG is usually 10-fold greater than the value of K_d determined by ITC.

4. Discussion

The ability to detect and elucidate detailed mechanisms of protein–ligand interactions enriches our comprehension of protein function. Indeed, many subcellular processes are inextricably linked to the ubiquitous and transient interactions between proteins and a variety of ligands (e.g., [34,35]). Predicting these interactions requires quantitative and qualitative prediction of the energetics of protein recognition and the dynamic interplay between ligand binding and altered protein function. The LacI system provides an example in which one binding pocket on the protein can bind a range of related molecules that have different allosteric effects on another aspect of protein function — in this case, DNA-binding.

Spectroscopic studies of ligand binding for most LacI effectors are precluded by the aromatic character of these compounds; however, a new vantage point is afforded by calorimetric measurements of thermodynamic properties for these sugar ligands binding to LacI. To assess whether this facile protocol has broader applications for future studies of ligand binding to the vast array of LacI allosteric variants (e.g., [18,32,33,36]), we explored conditions in which the monomer–monomer interface was perturbed by pH and/or assembly state. The wild-type interface changes significantly when inducer IPTG is bound [6,9,15,37]. Inversely, changes in the monomer–monomer subunit interface of LacI can substantially influence IPTG binding. For example, substituting K84 with L or A results in dramatic changes in IPTG binding kinetics [21], and V96E negates the anti-inducer influence of ONPF (H. Zhan, L. Swint-Kruse, and K.S. Matthews, unpublished results).

In the LacI variant with 11 C-terminal amino acids deleted ("LacI/−11", see Fig. 1) the tetramer assembly motif is entirely disrupted, and the monomer–monomer interface becomes less stable without alterations of amino acids forming these surfaces [19,24]. At low protein concentrations, this variant of LacI is in equilibrium between monomer and dimer [23]. Thus, if effector ligands exhibit different affinities for wild-type tetramer and LacI/−11, the sugar may well bind to one oligomeric state tighter than the other. The monomer–monomer interface also

appears to be the source of pH sensitivity for LacI–IPTG binding in Tris buffer [16,17]. This effect disappears in the monomeric form of the protein (created with the Y282D mutation) [17] but has not been localized to any particular residue.

The ITC measurements for ligand affinities agree substantially with literature values obtained by equilibrium dialysis and provide enthalpic data not previously accessible for the sugars with aromatic groups. For IPTG, the values in phosphate buffer are also similar to those measured by changes in fluorescence spectroscopy in Tris–HCl at neutral pH [16,32]. In the current experiments, IPTG binding is similar for tetramer, dimeric LacI/−11, and at both pHs examined; altered cooperativity and 10-fold diminished affinity at high pH seen in Tris–HCl buffer do not appear in the ITC/phosphate experiments, an observation confirmed by fluorescence measurement of affinity in phosphate buffer (Table 1). IPTG binding is driven by enthalpy, with a similar value for ΔH_{obs} for both oligomeric states and pHs examined. The only discrepancy from previous ITC studies is the enthalpic value at neutral pH [11]. In addition to being more facile than equilibrium dialysis, ITC measurements are sensitive to contaminating molecules and thus provide superior binding data.

Whereas IPTG values were reassuringly similar to affinities determined with other methods and in other buffers, ITC allowed us to detect new effects for the weak inducer, PhEG. First, the affinity of PhEG reported herein is ~4-fold tighter at pH 7.5 than that determined with equilibrium dialysis at pH 8.0, which is more comparable to the pH 9.2 value in this study. Second, the data for PhEG demonstrate that changes in pH and oligomeric state influence its binding properties and effects on operator binding. Indeed, operator release experiments show that PhEG is a weak inducer for LacI and LacI/−11, with substantial increase in the mid-point of release at elevated pH. For LacI tetramer at pH 9.2, significant binding is observed in the presence of PhEG. It is interesting to note the correlation between weak affinity and the weak allosteric effect on DNA-binding of PhEG compared to IPTG — one does not necessarily obligate the other.

Binding for anti-inducer ONPF is enhanced ~2- to 3-fold at pH 9.2, with a corresponding small increase in enthalpic forces over pH 7.5 for both tetramer and dimer forms. This sugar stabilizes LacI–operator binding for dimer and tetramer forms at both pH values. However, elevated pH appears to influence the monomer–monomer interface [17,19], and these changes may further facilitate the binding of ONPF. Interestingly, the neutral compound ONPG becomes an anti-inducer for LacI/−11 at pH 7.5 and for both proteins at pH 9.2, slightly enhancing the repressor–operator affinity. At elevated pH, both affinity and the enthalpic component of binding energy are slightly enhanced for ONPG.

We have generated an extensive series of LacI variants with changed allosteric properties [18,21,32,33,36,38] (H. Zhan, L. Swint-Kruse, J. Xu, and K.S. Matthews, unpublished results), based in part on *in silico* predictions of which residues participate in pathways of conformational change [39]. These LacI variants allow exploration of the ways allostery can be communicated through the fabric of the LacI core domain to alter the function of the DNA-binding domain. We have previously observed LacI mutants in which inducer IPTG binding does not correlate with operator release or for which inducer binding does not elicit the wild-type change in operator binding affinity [18,21,33,36,38]. Here, the weak inducer PhEG shows the same types of behaviors when binding to wild-type LacI. Thus, the ITC studies reported herein lay the foundation for new methods of exploring LacI allostery and provide novel tools for combination with effects created by amino acid side chain variation.

Further, studies of this prototypic gene regulator will allow extrapolation of protein ligand interactions, assembly, and allostery to other systems with structural homology. For example, the extracellular domains of class C G-protein coupled receptor (GPCR) family contains a fold that is similar to the region in LacI and binds to signal ligand (e.g., see [40]). Allosteric transmission of binding information is clearly an important feature of these crucial targets for therapeutic agents. Last, successful integration of interaction specificity and the thermodynamic profile of a recognition process may facilitate design of partners that form more stable complexes, with desired pharmaceutical outcomes [41–47].

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References

[1] F. Jacob, J. Monod, Genetic regulatory mechanisms in the synthesis of proteins, J. Mol. Biol. 3 (1961) 318–356.
[2] J.H. Miller, M.P. Calos, D. Galas, M. Hofer, D.E. Büchel, B. Müller-Hill, Genetic analysis of transpositions in the *lac* region of *Escherichia coli*, J. Mol. Biol. 144 (1980) 1–18.
[3] K.S. Matthews, J.C. Nichols, Lactose repressor protein: functional properties and structure, Prog. Nucleic Acid Res. Mol. Biol. 58 (1998) 127–164.
[4] W. Gilbert, B. Müller-Hill, Isolation of the *lac* repressor, Proc. Natl. Acad. Sci. U. S. A. 56 (1966) 1891–1898.
[5] A.D. Riggs, S. Bourgeois, On the assay, isolation and characterization of the *lac* repressor, J. Mol. Biol. 34 (1968) 361–364.
[6] M. Lewis, G. Chang, N.C. Horton, A. Kercher, H.C. Pace, M.A. Schumacher, R.G. Brennan, P. Lu, Crystal structure of the lactose operon repressor and its complexes with DNA and inducer, Science 271 (1996) 1247–1254.
[7] A.M. Friedman, T.O. Fischmann, T.A. Steitz, Crystal structure of *lac* repressor core tetramer and its implications for DNA looping, Science 268 (1995) 1721–1727.
[8] M.D. Barkley, A.D. Riggs, A. Jobe, S. Bourgeois, Interaction of effecting ligands with *lac* repressor and repressor–operator complex, Biochemistry 14 (1975) 1700–1712.
[9] C.E. Bell, M. Lewis, A closer view of the conformation of the Lac repressor bound to operator, Nat. Struct. Biol. 7 (2000) 209–214.

[10] S.L. Laiken, C.A. Gross, P.H. von Hippel, Equilibrium and kinetic studies of *Escherichia coli lac* repressor–inducer interactions, J. Mol. Biol. 66 (1972) 143–155.
[11] J. Donner, M.H. Caruthers, S.J. Gill, A calorimetric investigation of the interaction of the lac repressor with inducer, J. Biol. Chem. 257 (1982) 14826–14829.
[12] J.-C. Maurizot, M. Charlier, Circular dichroism studies of the binding of o-nitrophenyl-beta-D-fucoside and o-nitrophenyl-beta-D-galactoside to *Lac* repressor, Eur. J. Biochem. 79 (1977) 395–399.
[13] R. O'Brian, J.E. Ladbury, B.Z. Chowdhry, Protein–ligand interactions: hydrodynamics and calorimetry: a practical approach, in: B.Z. Chowdhry, S.E. Harding (Eds.), Isothermal Titration Calorimetry of Biomolecules, Oxford University Press, Oxford, 2001, pp. 263–286.
[14] B. Müller-Hill, H.V. Rickenberg, K. Wallenfels, Specificity of the induction of the enzymes of the *lac* operon in *Escherichia coli*, J. Mol. Biol. 10 (1964) 303–318.
[15] F. Boschelli, M.A. Jarema, P. Lu, Inducer and anti-inducer interactions with the *lac* repressor seen by nuclear magnetic resonance changes at tyrosines and tryptophans, J. Biol. Chem. 256 (1981) 11595–11599.
[16] B.E. Friedman, J.S. Olson, K.S. Matthews, Interaction of *lac* repressor with inducer. Kinetic and equilibrium measurements, J. Mol. Biol. 111 (1977) 27–39.
[17] T.J. Daly, K.S. Matthews, Characterization and modification of a monomeric mutant of the lactose repressor protein, Biochemistry 25 (1986) 5474–5478.
[18] C.M. Falcon, K.S. Matthews, Glycine insertion in the hinge region of lactose repressor protein alters DNA binding, J. Biol. Chem. 274 (1999) 30849–30857.
[19] J. Chen, K.S. Matthews, Deletion of lactose repressor carboxyl-terminal domain affects tetramer formation, J. Biol. Chem. 267 (1992) 13843–13850.
[20] R.B. O'Gorman, J.M. Rosenberg, O.B. Kallai, R.E. Dickerson, K. Itakura, A.D. Riggs, K.S. Matthews, Equilibrium binding of inducer to *lac* repressor·operator DNA complex, J. Biol. Chem. 255 (1980) 10107–10114.
[21] W.-I. Chang, J.S. Olson, K.S. Matthews, Lysine 84 is at the subunit interface of *lac* repressor protein, J. Biol. Chem. 268 (1993) 17613–17622.
[22] M.D. Barkley, P.A. Lewis, G.E. Sullivan, Ion effects on the *lac* repressor–operator equilibrium, Biochemistry 20 (1981) 3842–3851.
[23] J. Chen, K.S. Matthews, Subunit dissociation affects DNA binding in a dimeric *lac* repressor produced by C-terminal deletion, Biochemistry 33 (1994) 8728–8735.
[24] M. Brenowitz, N. Mandal, A. Pickar, E. Jamison, S. Adhya, DNA-binding properties of a *lac* repressor mutant incapable of forming tetramers, J. Biol. Chem. 266 (1991) 1281–1288.
[25] D.R. Wycuff, K.S. Matthews, Generation of an AraC-*ara*BAD promoter-regulated T7 expression system, Anal. Biochem. 277 (2000) 67–73.
[26] R.B. O'Gorman, M. Dunaway, K.S. Matthews, DNA binding characteristics of lactose repressor and the trypsin-resistant core repressor, J. Biol. Chem. 255 (1980) 10100–10106.
[27] A.D. Riggs, S. Bourgeois, R.F. Newby, M. Cohn, DNA binding of the *lac* repressor, J. Mol. Biol. 34 (1968) 365–368.
[28] I. Wong, T.M. Lohman, A double-filter method for nitrocellulose-filter binding: application to protein–nucleic acid interactions, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5428–5432.
[29] W. Gilbert, A. Maxam, The nucleotide sequence of the *lac* operator, Proc. Natl. Acad. Sci. U. S. A. 70 (1973) 3581–3584.
[30] B.E. Friedman, J.S. Olson, K.S. Matthews, Kinetic studies of inducer binding to lactose repressor protein, J. Biol. Chem. 251 (1976) 1171–1174.
[31] T. Arakawa, S.N. Timasheff, Stabilization of protein structure by sugars, Biochemistry 21 (1982) 6536–6544.
[32] L. Swint-Kruse, H. Zhan, B.M. Fairbanks, A. Maheshwari, K.S. Matthews, Perturbation from a distance: mutations that alter LacI function through long-range effects, Biochemistry 42 (2003) 14004–14016.
[33] L. Swint-Kruse, H. Zhan, K.S. Matthews, Integrated insights from simulation, experiment, and mutational analysis yield new details of LacI function, Biochemistry 44 (2005) 11201–11213.
[34] A.-C. Gavin, M. Bösche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, M. Rick, A.M. Michon, C.-M. Cruciat, M. Remor, C. Höfert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M.-A. Heurtier, R.R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, G. Superti-Furga, Functional organization of the yeast proteome by systematic analysis of protein complexes, Nature 415 (2002) 141–147.
[35] Y. Ho, A. Gruhler, A. Heilbut, G.D. Bader, L. Moore, S.-L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A.R. Willems, H. Sassi, P.A. Nielsen, K.J. Rasmussen, J.R. Andersen, L.E. Johansen, L.H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B.D. Sorensen, J. Matthiesen, R.C. Hendrickson, F. Gleeson, T. Pawson, M.F. Moran, D. Durocher, M. Mann, C.W.V. Hogue, D. Figeys, M. Tyers, Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry, Nature 415 (2002) 180–183.
[36] J.K. Barry, K.S. Matthews, Substitutions at histidine 74 and aspartate 278 alter ligand binding and allostery in lactose repressor protein, Biochemistry 38 (1999) 3579–3590.
[37] L. Swint-Kruse, Using networks to identify fine structural differences between functionally distinct protein states, Biochemistry 43 (2004) 10886–10895.
[38] H. Zhan, L. Swint-Kruse, K.S. Matthews, Extrinsic interactions dominate helical propensity in coupled binding and folding of lactose repressor protein hinge helix, Biochemistry 45 (2006) 5896–5906.
[39] T.C. Flynn, L. Swint-Kruse, Y. Kong, C. Booth, K.S. Matthews, J. Ma, Allosteric transition pathways in the lactose repressor protein core domains: asymmetric motions in homodimer, Protein Sci. 12 (2003) 2523–2541.
[40] C.B. Felder, R.C. Graul, A.Y. Lee, H.-P. Merkle, W. Sadee, The Venus flytrap of periplasmic binding proteins: an ancient protein module present in multiple drug receptors, AAPS PharmSci. 1 (1999) (Article 2, http://www.aapspharmsci.org).
[41] C.L. Vizcarra, S.L. Mayo, Electrostatics in computational protein design, Curr. Opin.Chem. Biol. 9 (2005) 622–626.
[42] G. Cheng, B. Qian, R. Samudrala, D. Baker, Improvement in protein functional site prediction by distinguishing structural and functional constraints on protein family evolution using computational design, Nucleic Acids Res. 33 (2005) 5861–5867.
[43] H.W. Hellinga, Rational protein design: combining theory and experiment, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10015–10017.
[44] L.L. Looger, M.A. Dwyer, J.J. Smith, H.W. Hellinga, Computational design of receptor and sensor proteins with novel functions, Nature 423 (2003) 185–190.
[45] M.A. Dwyer, H.W. Hellinga, Periplasmic binding proteins: a versatile superfamily for protein engineering, Curr. Opin. Struct. Biol. 14 (2004) 495–504.
[46] M.A. Dwyer, L.L. Looger, H.W. Hellinga, Computational design of a biologically active enzyme, Science 304 (2004) 1967–1971.
[47] H.-S. Park, S.-H. Nam, J.K. Lee, C.N. Yoon, B. Mannervik, S.J. Benkovic, H.-S. Kim, Design and evolution of new catalytic activity with an existing protein scaffold, Science 311 (2006) 535–538.