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Functional Consequences of Exchanging Domains Between LacI and PurR are Mediated by the Intervening Linker Sequence

Sudheer Tungtur¹, Susan M. Egan², and Liskin Swint-Kruse^{1,*}

¹ Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, Kansas City, Kansas 66160

² Department of Molecular Biosciences, The University of Kansas–Lawrence, Lawrence, Kansas 66045

Abstract

Homologue function can be differentiated by changing residues that affect binding sites or long-range interactions. LacI and PurR are two proteins that represent the LacI/GalR family (>500 members) of bacterial transcription regulators. All members have distinct DNA-binding and regulatory domains linked by ~18 amino acids. Each homologue has specificity for different DNA and regulatory effector ligands; LacI and PurR also exhibit differences in allosteric communication between DNA and effector binding sites. A comparative study of LacI and PurR suggested that alterations in the interface between the regulatory domain and linker are important for differentiating their functions. Four residues (equivalent to LacI positions 48, 55, 58, and 61) appear particularly important for creating a unique interface and were predicted to be necessary for allosteric regulation. However, nearby residues in the linker interact with DNA ligand. Thus, differences observed in interactions between linker and regulatory domain may be the *cause* of altered function or an *effect* of the two proteins binding different DNA ligands. To separate these possibilities, we created a chimeric protein with the LacI DNA-binding domain/linker and the PurR regulatory domain (LLhP). If the interface requires homologue-specific interactions in order to propagate the signal from effector binding, then LLhP repression should not be allosterically regulated by effector binding. Experiments show that LLhP is capable of repression from *lacO^I* and, contrary to expectation, allosteric response is intact. Further, restoring the potential for PurR-like interactions via substitutions in the LLhP linker tends to *diminish* repression. These effects are especially pronounced for residues 58 and 61. Clearly, binding affinity of LLhP for the *lacO^I* DNA site is sensitive to long-range changes in the linker. This result also raises the possibility that mutations at positions 58 and 61 co-evolved with changes in the DNA-binding site. In addition, repression measured in the absence and presence of effector ligand shows that allosteric response increases for several LLhP variants with substitutions at positions 48 and 55. Thus, while side chain variation at these sites does not generally dictate the presence or absence of allostery, the nature of the amino acid can modulate the response to effector.

Keywords

lactose repressor protein; purine repressor protein; DNA-binding; allostery; domain linker; transcription regulation; LacI/GalR; specificity determinants

*Correspondence to: Liskin Swint-Kruse, Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, Kansas City, Kansas 66160. E-mail: lswint-kruse@kumc.edu.

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INTRODUCTION

Members of LacI/GalR family of transcription regulators comprise a huge family of proteins, with >500 members distributed among virtually all bacterial species. *Escherichia coli* contains 16 paralogues, including LacI and PurR (lactose and purine repressor proteins, respectively). Many family members coordinate transcription with changes in environmental/metabolic conditions, usually through direct binding of a related small effector molecule (i.e. LacI binds allolactose; PurR binds hypoxanthine or guanine). The operator DNA sequences recognized by these proteins also differ. DNA-binding affinity is usually diminished when a family member binds its cognate effector (LacI is naturally induced by allolactose), but some proteins attain increased affinity for DNA in the presence of small molecule (hypoxanthine and guanine are co-repressors for PurR), reviewed in Ref. 1. These different functions are clearly accomplished by variation in effector and DNA-binding sites, but variation in other regions of the protein also can convey unique function. One region that potentially makes long-range functional contributions is the linker that joins the two functional domains of LacI/GalR proteins [Fig. 1(A)].²

The linker was first characterized by its high proteolytic susceptibility, which allowed isolation of two functional domains for LacI and PurR^{6–9}; homologous domains are present in the larger LacI/GalR family.^{1,10,11} Each domain contains a binding site for one ligand: The N-terminal domain binds DNA and thereby affects transcription; the C-terminal regulatory domain binds the effector molecule to allosterically regulate DNA affinity. When structures of LacI/GalR proteins were solved, one striking find was that the linker is not a passive connection between the functional domains.^{4,5,12–15} Indeed, the functional domains do not directly contact each other. Instead, the 18 amino acid linker is sandwiched between the two domains, making extensive contacts to both (Fig. 2).

Another feature of the linker is a coil-to-helix transition of the central ~10 amino acids (hinge helix) that is coincident with DNA binding. Although the coil conformation has not been directly observed in full-length protein, a large body of work is consistent with this region being unstructured and highly mobile for LacI and PurR in the absence of DNA ligand.^{12, 17–23} When folded, the hinge helix forms additional contacts with DNA and with the hinge helix of the other monomer in the dimer.^{4,12,14,15,17–26} Thus, the linker region is highly connected and appears to have a very important role in modulating the conformational changes that are central to transcription regulation.

A comparative study of LacI and PurR indicated that several positions in the linker regions are (1) structurally different, (2) functionally sensitive to mutations, and (3) highly *variable* in sequence across the family [Fig. 1(A, C), Fig. 2, Table I]. These observations resulted in the hypothesis that variation at these sites provides opportunity to evolve unique properties for individual family members.² Subsequent results and structures for family member CcpA are consistent with this hypothesis.^{31,32} Several of these linker positions contact the regulatory domain, which precipitated the hypothesis that these sites allow allosteric communication between the DNA-binding and regulatory domains. However, the proximity of the DNA-binding site raises the possibility that differences between LacI and PurR structures are simply the result of binding different operator ligands and do not directly contribute to functional differences between LacI/GalR family members.

Discrimination between these two possibilities requires a series of proteins that bind the same DNA ligand. To that end, we have constructed chimeric proteins, using the LacI DNA-binding domain joined to the regulatory domains of various members of the LacI/GalR family [for example, Fig. 1(B)]. In these contexts, the sequence of the linker can be further varied. This

work reports comparison of repressor function for LacI, a LacI:PurR chimera (LLhP^{*}), and LLhP variants with substitutions at the sites predicted to convey allostery.

MATERIALS AND METHODS

Construction of LLhP

Chimeras utilize the LacI DNA binding domain because constitutive LacI overexpression is very well tolerated in *E. coli*. In contrast, overexpression of intact PurR protein is toxic,³³ probably due to the metabolic importance of the operons regulated by this protein. We presumed that any chimera with a PurR DNA-binding domain would have similar toxicity while those with a LacI DNA-binding domain would be well-tolerated. For this work, the LacI DNA-binding domains were joined to the LacI linker, then to the PurR regulatory domain [LLhP; Fig. 1(B)]. Surprisingly, LLhP exhibited toxicity in some cell strains (see later), which could indicate altered DNA-binding specificity of the LacI DNA-binding domain.

Chimera construction relies upon accurate identification of the ends of the linker sequence and should not disrupt the structure of the regulatory domain. For LLhP, identifying the end of the C-linker and the start of the regulatory domain was difficult. In LacI, position 63 is the first residue in a β -strand that is part of a β -sheet that is central to the subdomain structure. Position 62 in LacI is extremely tolerant of substitutions—none of 14 substitutions had a measurable effect on phenotype.³⁴ Based upon this information, we decided to join the chimeric protein between residues 61 and 62.

All primers for mutagenesis were purchased from Integrated DNA Technology (Coralville, IA) or the Biotechnology Support Center at KUMC. DNA sequencing was carried out by the KUMC Biotechnology Support Center or Northwoods DNA (Solway, MN). Chimeric protein LLhP comprises LacI 1–61 and PurR 60–343 (note that PurR numbers differ by 2 in this region for homologous positions; Table I). The *lacI* gene and promoter regions are contained on the pLS1 plasmid.³⁵ pLS1 also contains a Bsu36I site downstream of the gene encoding LacI, which was utilized for LLhP construction. To create LLhP, we first mutated pLS1 to create an AfeI site at codon 62 using site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA), using the primers 5'GCGGGCAAACAGAGCGCTCTGATTGGCGTTGC3' and 5'GCAACGCCAATCAGAGCGCTCTGTTTGGCCGC3'. The resulting plasmid was called pLac-AfeI.

The desired fragment of PurR (amino acids 60–343) was amplified from *E. coli* DH5 α (Invitrogen, Carlsbad, CA) using colony PCR (PCR Mastermix, Promega, Madison, WI). Primers 5'GCCTGAAGGTTAACCAATTTAAATCT-ATCGGTTTGCTGGCG3' and 5'CCATCAGAAGCTCTA-CCTCAGGTTATTAACGACGATAGTCG3' were used to amplify the PurR regulatory domain with (1) a SmaI site at codon 60, (2) an additional stop codon, and (3) a Bsu36I site following the second stop codon. PCR products were ligated into pGemT vector (Promega, Madison, WI). White colonies were grown overnight in 3 mL cultures of 2 $\times$ YT and plasmid DNA purified with QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) or Quantum Prep Plasmid Mini-prep Kit (Bio-rad, Chicago, IL). Plasmids were screened with restriction digestion to identify the appropriate insert; if positive, the insert was sequenced using primers to the SP6 and T7 sites on pGemT vector.

To create pLLhP plasmid, pLac-Afe and the pGemT plasmid containing the appropriate insert were cut with SmaI and Bsu36I, gel-purified, and ligated after the two fragments were co-

*Nomenclature for the chimeras follows the general scheme of XYhZ, where X indicates the parent protein of the DNA-binding domain, Yh indicates the parent protein of the linker region (and contains a *hinge helix*), and Z indicates the parent protein of the regulatory domain.

precipitated with ethanol. The entire coding region of the gene was sequenced using the primers 5'GCTCGAGGTCGACGGATCCC3' and 5'CATCAACATTAAATGTGAGC3' (for sequencing codons 1 to ~220 and ~196 to “stop”, respectively). Any mutation that arose during the formation of the chimeras (usually during amplification of PurR genes from *E. coli*, which used a low-fidelity polymerase) were corrected so that the final sequence matched the published LacI/PurR sequences^{29,36} using site-directed mutagenesis as earlier. The final sequence was confirmed by sequencing the entire coding region.

The resulting protein sequence for LLhP is shown below, with the linker sequence underlined:

MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAK-
 TREKVEAAMAELNYIPNRVAQQLAGKQS
 KSIGLLATSSEAAAYFAEIIIEAVEKNCFQKGYTLILG-
 NAWNNLEKQRAYLSMMAQKRVDG
 LLVMCSEYPELLAMLEEYRHIPMVVMDWGEA-
 KADFTDAVIDNAFEGGYMAGRYLIERGH
 REIGVIPGLERNTGAGRLAGFMKAMEEAMIKVPES-
 WIVQGDPEPESGYRAMQQILSQPH
 RPTAVFCGGDIMAMGALCAADEMGLRVPQDVSLI-
 GYDNVRNARYFTPALTTIHQPKDSL
 ETAFNMLLDRIVNKREEPQSIEVHPRIERRS-
 VADGPFRDYRR

To aid repeatability in phenotypic screening, genes encoding the chimeras were subcloned into a low copy plasmid pHG16537 using the following strategy: EcoRI was used to cut the pLS1-derived plasmid pLLhP on either side of the gene for the regulatory protein (the exact position of the downstream EcoRI site is unknown). The appropriate fragment was gel purified and ligated into the single EcoRI site of pHG165. Subcloning was verified by the formation ~4.9 kb plasmid that has a ~1 kb dropout band upon digestion with SacI and ScaI. Sequences of subcloned genes were verified by sequencing the entire coding region.

Designed and Random Site-directed Mutagenesis

Variants of LLhP were created with site-directed mutagenesis (Quikchange, Stratagene), using the primers listed in Table II. For random mutagenesis, primers were degenerate at the appropriate codon (“NNN”), and the Quikchange protocol was modified so that, instead of plating transformed cells at the end of the procedure, cells were transferred to 3 mL LB media, grown overnight, and used to purify a sample of mixed plasmid. This randomly mutated DNA was transformed in *E. coli* 3.300 cells for phenotypic screening (see later). DNA was purified from representative blue, light blue, and white colonies. The entire gene was sequenced to determine which amino acid was encoded at the mutated position and to verify that no unexpected mutations were created.

Phenotypic Analysis—Plate and Liquid Culture Assays of β -Galactosidase Activity Plate assays^{35,38,39}

Guanine and hypoxanthine are alternative co-repressors for wild-type PurR; binding co-repressor enhances PurR repression.^{8,40} We expected that LLhP would behave similarly if capable of an allosteric conformational change. LLhP phenotypes were determined using *E.*

coli 3.300 cells (*E. coli* Genetic Stock Center, Yale University), which have an interrupted *lacI* gene but an intact *lacZYA* operon controlled by the operator sequence *lacO*^L.⁴¹ Assays utilized the blue–white indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal) 39 in standard LB plates with 100 μg/mL ampicillin, incubated overnight at 35°C. If present, adenine was 25 μg/mL⁴²; this molecule is metabolized to co-repressor hypoxanthine. In this assay, white colonies express protein capable of repressing the *lacZYA* operon by binding *lacO*^L; blue colonies express protein that cannot repress transcription of these genes.

Liquid culture β-galactosidase assay^{39,43,44}

Liquid culture assays are more sensitive than plate assays and can discriminate a broader range of functional differences. Starter cultures of *E. coli* 3.300 cells expressing LLhP variants were inoculated in 3 mL 2×YT broth containing 100 μg/mL of ampicillin. Freshly-transformed colonies (less than a day old) were used as the inoculum and were grown for ~7 h at 35°C. Forty microliter of this starter culture was then used to inoculate 2.5 mL of overnight medium, which was grown for 16–18 h. Overnight medium consisted of 1× MOPS buffered minimal medium^{43,44} (morpholinopropanesulfonic acid; Teknova-Hollister, CA), with 0.04% glycerol as the carbon source, 1.32 mM dibasic potassium phosphate, 10 mM NaHCO₃, 0.2% casamino acids, 0.0025% thiamine, and 100 μg/mL ampicillin. Each overnight culture (100 μL) was used to inoculate two 10 mL samples of growth medium in baffled 125 mL Erlenmeyer flasks. Growth medium consisted of 1× MOPS media, 0.8% glycerol, 1.32 mM dibasic potassium phosphate, 10 mM NaHCO₃, 0.2% casamino acids, 0.00125% of thiamine, and 100 μg/mL of ampicillin. Of the two flasks, one contained 25 μg/mL of adenine. Cultures were grown at 37°C with vigorous shaking until an OD₆₀₀ of 0.4 was reached; care was taken not to exceed this value. Cells were harvested by centrifugation for 10 min at 7650g at 4°C. Cells were resuspended in 4 mL of Working Buffer (100 mL of Enzyme Assay Buffer with 0.4 mM DTT). Enzyme Assay Buffer was 61 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄. The OD₆₀₀ of each cell suspension was measured; optimal values after resuspension were near 1.

Cell suspensions (10 μL–1 mL, iteratively determined by protein activity) were mixed with working buffer for a final volume of 1 mL in a 100 × 13 mm² enzyme assay tube. Two samples were created from each cell culture, one with 2× the volume of cell suspension compared to the other. Diluted cell suspensions were permeabilized by adding 20 μL of Polymixin B (RPI, Mt.Prospect, IL), vortexing for 5 s, and incubating at room temperature for 5 min. The colorimetric assay was initiated by adding 200 μL of substrate *o*-nitrophenyl-β-D-galactopyranoside (RPI, Mt.Prospect, IL). Samples were incubated at room temperature until they became visibly yellow. The assay reactions were stopped by adding 500 μL of 1M sodium carbonate and the elapsed time was recorded. Reactions were centrifuged in a Hermle 1200 M/H at full speed for 10 min to pellet the cells prior to determining OD₄₂₀ on a Carey 50BIO (Varian). The β-galactosidase activity was calculated using the formula

$$\text{Units} = 1000[\text{OD}_{420}] / [t \times v \times \text{OD}_{600}]$$

where *t* is elapsed time in minutes and *V* is the volume of overnight culture in mL. Wild-type LacI –/+ inducer IPTG was always assayed in parallel to provide an internal calibration point (values for unmutated LLhP were too low to serve this function). The number of β-galactosidase units measured for LacI in the presence of IPTG was set to 100 and used to normalize the LLhP samples. The duplicate measurements were made at least three and up to six separate times for each variant, always from a fresh transformation of plasmid into *E. coli* 3.300 cells.

RESULTS AND DISCUSSION

Bioinformatics and mutational analyses suggests that a subset of linker residues that interact with the regulatory domain endow LacI/GalR family members with unique functions² [Fig. 1 (C), Table I]. These residues are located on three distinct regions of the linker: One of the predicted positions (LacI a.a. 48) is in the unstructured *N-linker* that connects the hinge helix to the N-terminal DNA-binding domain. Another position (LacI a.a. 55) is in the central *hinge helix* that undergoes a coil-to-helix transition upon DNA-binding. The last two positions (LacI a.a. 58 and 61) are in the unstructured *C-linker* that connects the hinge helix to the regulatory domain. However, in addition to these residues that interact with the regulatory domain, other residues in the hinge helix also interact with DNA ligand. Thus, interpretation of LacI and PurR differences is limited by the fact that the DNA-ligand is different for these two family members. To parse the contributions of protein–protein and protein-DNA interactions to functional differences of homologues, we have constructed chimeric proteins that have a common DNA-binding domain and linker. LLhP has the LacI DNA-binding domain, the entire LacI linker, and the PurR regulatory domain.

Both LacI and PurR repress transcription when bound to DNA *in vivo*. However, these homologues have opposite allosteric response to binding effector molecules. LacI has higher DNA-binding affinity in the *absence* of inducers; affinity for DNA is diminished in the presence of either allolactose or IPTG.^{45,46} PurR binds DNA more tightly in the *presence* of co-repressor (hypoxanthine or guanine) than in the absence of the small molecules.^{47,48} Both behaviors result from the conformational change of the regulatory domain as it binds effector molecule.^{12,14} We originally expected that LLhP construction would disrupt the interface between the linker and regulatory domain, thereby abolishing allostery. Since co-repressor is required for high affinity binding of PurR, we hypothesized that LLhP might be trapped in a state with low affinity for DNA, and thus be unable to repress transcription. We further predicted that any LLhP variants with restored allosteric response to effector would respond to co-repressor like wild-type PurR, with increased DNA-binding and transcription repression.

Phenotype of LLhP

In vivo phenotypic assays were used to assay the ability of LLhP to repress transcription and respond to co-repressor. Contrary to our expectations, the LLhP phenotype on indicator plates of either LB or minimal media^{*} was white in both the absence and presence of effector, suggesting that this chimera always repressed transcription (Table III). These phenotypes were consistent regardless of whether the LLhP gene was carried on a variant of the high-copy pLS1 plasmid or the lower-copy plasmid derived from pHG165. Since plate assay results did not distinguish the enhanced LLhP function in the presence of co-repressor that was detected using purified repressor (unpublished results), we turned to a more sensitive, quantitative liquid culture assay of β -galactosidase activity.^{39,43,44} Results demonstrate approximately twofold increase in LLhP repression in the presence of co-repressor (see Fig. 3). Despite the *in vitro* similarities for LacI and LLhP binding *lacO*^I (unpublished results), *in vivo* expression of LacI from the pHG165 plasmid resulted in tighter repression than LLhP on the same plasmid. Tighter *in vivo* LacI repression can most likely be attributed to tetrameric LacI binding two operator sequences naturally present in the *lac* operon[†].

^{*}To rule out the possibility of endogenous co-repressor in LB media.

[†]LacI tetramer formation derives from an additional C-terminal domain that allows a dimer of dimers to form.^{12,51–53} LacI tetramer can simultaneously bind two operator sequences that are naturally present in the *lac* operon (the source of the reporter gene in *E. coli* 3.300 cells). Creation of the looped *lacO*²-LacI-*lacO*^I complex results in tighter repression.^{54–58} PurR and LLhP variants lack a tetramerization domain; other evidence confirms that PurR is a dimer.^{8,59} Dimeric LLhP should only bind one operator sequence and is unable to form repression loops.

Unexpectedly, LLhP showed *in vivo* behavior consistent with toxicity of this protein. In *E. coli* 3.300 cells, re-plated cells subsequent to transformation with pLLhP lost their ability to repress transcription of the reporter gene, although both the plasmid and the LLhP gene remained intact. TB1 cells transformed with pLLhP yielded no detectable protein, and pLLhP transformations into the BLIM strain60 never produced colonies. This apparent toxicity was not rescued by adenine. Our resulting hypothesis is that LLhP binds a DNA operator critical to *E. coli* that is not normally recognized by wild-type LacI. Thus, changing interactions between the linker and the regulatory domain may affect LLhP specificity for DNA ligands. Alternatively, the PurR regulatory domain may be the source of toxicity.

Function of LLhP Linker Variants

Earlier structure/function comparisons of LacI and PurR predicted that linker positions 48, 55, 58, and 61* may convey unique function to LacI/GalR family members.² All of these positions interact with the top surface of the regulatory domain. More specifically, positions 48 and 55 make interactions in LacI that are absent in PurR [Fig. 2(A, B); red lines]. Chimeric LLhP may also form these interactions, given that the region around the relevant residues on the regulatory domain is moderately conserved in the two parent proteins (LacI residues 112–120, Table I); and residue 55 forms helix–helix interactions with position 52 on the other monomer, which is conserved as valine in both LacI and PurR. Mutagenesis of positions 48 and 55 from the LacI to the PurR sequence was thus expected to abolish function associated with their interactions with the regulatory domain.

In contrast, the nature of the residue at LacI/LLhP position 58 (PurR 56) appears to change the trajectory of the C-linker; the result is that positions PurR 58/59 make more contacts with the regulatory domain than their LacI equivalents at positions 60/61 [Fig. 2(A, B); blue lines]. The nature of the side chain at PurR position 59 may also facilitate specific cross-domain interactions.² Chimera construction of LLhP would presumably interrupt the C-linker/regulatory domain interface found in PurR and affect the relevant functions. In this case, mutagenesis of the LLhP C-linker positions to the residues found in PurR was expected to restore function associated with the interactions.

To test these hypotheses, we made three types of mutations in the LLhP linker: The first changed the residue from the amino acid that occurs naturally in LacI to that of PurR. The second was site-directed, random mutagenesis of the same position, to determine which amino acids do and which do not allow function at these sites. The third swapped combinations of mutations (e.g. the entire C-linker) between LacI-derived LLhP and PurR sequences. All LLhP variants were characterized by their phenotypes on indicator plates; a subset of variants was also characterized by the more sensitive liquid culture method. Results are summarized in Table III, Figures 3 and 4.

N-Linker Position 48

Both LacI and LLhP have an isoleucine at position 48; PurR has a serine. Both types of side chains interact with the regulatory domain, but the larger isoleucine forms more interactions in LacI than does serine in PurR. The ability of isoleucine to interact with the regulatory domain and strengthen the interface may be the source of the extremely tight DNA binding observed for LLhP. The LacI to PurR mutation in LLhP/I48S—which is predicted to abrogate the contact with the regulatory domain—diminishes transcription repression (see Fig 3). Allosteric response to co-repressor is intact and may be somewhat enhanced (see Fig. 4).

*LacI and LLhP numbering; PurR numbering differs by 2. (LacI 48 = PurR 46)

Random mutagenesis of position 48 shows that this position tolerates a number of substitutions. Several of the substitutions with greatly diminished function are easy to rationalize: Diminished repression of I48G may be due to increased flexibility of the N-linker increasing the motions of the DNA-binding domain⁶¹; I48R may experience electrostatic repulsion with the K and R that are present in the PurR regulatory domain near position 118. Like I48S, the variants with I48H, I48V, and I48T increase allosteric response by weakening repression in the absence of co-repressor while having more moderate effects on repression in the presence of co-repressor. Surprisingly, the “conservative” substitution I48L cannot repress transcription. In general, substitutions appear to have similar effects on LLhP and wild-type LacI functions (Table III).

Can these mutational results be rationalized with one structural framework? So far, we have considered that the role of position 48 to affinity and allostery comes from strengthening interactions with the regulatory domain. All but the results for I48L are consistent with this hypothesis. An extension of this idea is suggested by comparing structures of full-length and truncated (DNA-binding domain with partial linkers) LacI/GalR proteins and may also explain the behavior observed for I48L.²

In the full-length DNA-bound structure of LacI, I48 interacts with the regulatory domain. In the truncated structure, the side chain of I48 exhibits a very different trajectory and interacts with a hydrophobic region of the DNA-binding domain.^{2,62} Our first tendency was to dismiss the alternate side chain positions as an artificial consequence of truncating the protein. However, if the hinge helices are only folded in the presence of cognate DNA, the distance/orientation between residue 48 and the regulatory domain need not be constrained in the apo-protein, and the side chain of I48 may indeed interact with the DNA-binding domain[†]. Thus, the side chain at position 48 could “toggle” between interacting with either of the two functional domains.

Notably, the hydrophobic interaction region in the DNA-binding domain is generally conserved throughout the LacI/GalR proteins, providing opportunity for this toggle to contribute to allostery across the family, with adjustments provided by the nature of the side chain at position 48. In PurR, the serine at this position is too short to make similar contacts with the DNA-binding domain,¹⁸ but the structure of the truncated FruR DNA-binding domain shows a similar interaction between the histidine at this position and the DNA-binding domain.⁶³ This hypothesis also provides a rationale for LLhP/I48L: The loss of repression in this variant could be explained if the leucine side chain interacts very strongly with the DNA-binding domain and traps the protein in a conformation with low DNA-binding affinity.

Hinge Helix Position 55

LacI and LLhP have glutamine at position 55 of the hinge helix; PurR has serine in this position. In LacI, this position forms contacts with other residues in the hinge helix and residues in the regulatory domain that are absent in PurR (see Fig. 2). Thus, in addition to the interaction with the regulatory domain, the side chain of position 55 may affect helix–helix packing⁶⁴ and helical propensity, since this hinge helix appears to be unfolded in the absence of DNA. The Q55S substitution has diminished transcription repression, but the allosteric response may show a small increase (Figs. 3 and 4). Two other substitutions—Q55T and Q55V—exhibit significantly higher *in vivo* allosteric response. Effects of Q55T are from increased repression in the presence of adenine. Consequences of Q55V arise from lost repression without co-repressor. These “shorter” side chains are not expected to contact the regulatory domain. We considered whether enhanced allostery correlates with decreased intrinsic helical propensity: The intrinsic propensities of S, T, and V chains in the C3 position of a helix are very low⁴⁹

[†]No structure is available of this form of the full-length protein.

(Table III). However, T has an opposite functional affect to V and S, and increasing intrinsic propensity with Q55A does not impart increased affinity or altered allostery to LLhP. Thus, as was observed for other LacI linker variants,⁶⁵ intrinsic propensity may be superseded by other structural factors such as helix/helix packing. In general, substitutions at position 55 appear to have similar functional effects in LLhP and wild-type LacI (Table III).

C-Linker Residues 58 and 61

The C-linkers of LacI and PurR are quite different from each other, both in the types of residues and in the interactions made with the regulatory domain [Figs. 1(A) and 2]. Since the LacI residues in LLhP positions 58 and 61 presumably do not form interactions equivalent to those seen in wild-type PurR, we anticipated that substitutions with PurR residues would restore their contacts and “improve” function. However, both G58V and S61T clearly diminished repression. SDS-PAGE indicated that soluble LLhP variants were expressed in all cases. Random mutagenesis identified no substitutions that were functional at position 58 and only one—cysteine—that allowed moderate repression at position 61. (Although the plate assay indicated moderate function for leucine, methionine, and glutamine at 61, these substitutions were less effective in the more sensitive liquid culture assay.) LLhP variants with substitutions at position 61 may have residual function, since repression was always tighter in the presence of co-repressor. Notably, the effects of substitutions at position 61 in LLhP are fundamentally different than effects of substitutions at this position in wild-type LacI, which most frequently retain repression but are unable to respond to effector ligand.³⁴ In fact, LacI/S61L has increased DNA-binding affinity,⁵⁰ whereas LLhP/S61L has diminished repression, presumably as a result of diminished affinity for DNA. Substitutions in LLhP at position 58 generally showed no allosteric response. The G58R mutation also had different functional outcomes in LLhP and LacI.

Since the type of residue at 58 determines the length of the hinge helix and the trajectory of the C-linker [Fig. 1(A)], changes at this position probably affect the ability of residues 59–61 to interact with the regulatory domain. Thus, we also determined the effects of a double mutant (G58V/S61T) and one in which all residues of the C-linker were restored to the PurR sequence (LLhP/Cswap). Neither construct was capable of repressing transcription from *lacO^I*, and LLhP/Cswap appears to have abolished allosteric response (Figs. 3 and 4). Likewise, the LLhP/Quad variant (I48S/Q55S/G58V/S61T)—which theoretically allows all potential PurR interface interactions to form and removes all putative LacI interactions—does not repress or exhibit allosteric response to co-repressor. These results generate a range of possibilities: Do the Clinker interactions not form as expected in LLhP variants? Alternatively, the LacI structure does *not* have the interactions between the C-linker and regulatory domain—is the LacI C-linker sequence unique in this respect? Continuing along those lines, if the LLhP C-linker substitutions permit interactions with the PurR regulatory domain, perhaps they trap the protein in a conformation that does not allow binding to *lacO^I*. Alternatively, interactions with the regulatory domain may diminish conformational flexibility of the C-linker, which may be required to bind the asymmetric *lacO^I*. The latter is suggested by LacI variants Q60G, which enhances *lacO^I* binding affinity, and Q60P, which diminishes affinity.⁶⁶ Similarly, comparison of the truncated LacI DNA-binding domain structures in complex with *lacO^I* and *lacO^{sym}* indicates that flexibility in the N-linker is necessary to accommodate the asymmetry of *lacO^I*.²⁶ Experiments are underway to determine if LLhP C-linker variants can bind other variant operator ligands.

CONCLUSIONS

Protein function can be altered by changing amino acids that (1) participate in ligand binding or (2) make long-range contributions to function.^{67–70} By extension, homologues can evolve

unique functions via mutations in both class of residues.⁷¹ Intuitively, allostery is expected to be affected by regions of the protein outside of binding sites, and we originally anticipated all functional effects in chimeras and variants would be allosteric. While we did detect allosteric consequences, we also observed unexpected and dramatic changes in affinity when substitutions were made at positions 58 and 61.

Recent theories of allostery have expanded beyond interconversion between a few protein conformations and now include shifts between pre-existing conformational ensembles, through-protein pathways of communication, and changes in dynamic motions.^{72–76} LacI and PurR allostery are known to involve at least two protein conformations,^{4,14} perhaps more.⁷⁷ When either protein is bound to DNA, the interface between the linker and the regulatory domain is formed (note that PurR also has co-repressor effector bound). Presumably a chimeric interface is formed when LLhP forms a complex with DNA and co-repressor. In the absence of DNA, these interfaces are disrupted for LacI and PurR.^{4,14} In the conformation with low affinity for DNA (LacI + IPTG, PurR without co-repressor, and LLhP without co-repressor), these proteins still bind DNA but the conformation of the hinge helix or linker interface remains unknown. Another “unknown” is the structural consequence of linker substitutions: They may affect allostery by altering a pre-existing structural ensemble or by allowing new conformations. Further study of LLhP allosteric mutants will facilitate comparison of the range of allosteric mechanisms that occurs within the LacI/GalR family.

Some insights into the mechanism of allostery are gained from current results: Since the linker interface forms under conditions with highest repression, we originally expected all functional changes to evince in these circumstances. Instead, the allosteric effects of varying LLhP positions 48 and 55 often occurs because function in the low repression state (no adenine) worsens more than in the high repression condition. We envision two possible explanations for this observation: Either the linker-regulatory domain interface also forms in the low-repression condition, or these side chains make critical interactions with regions of the protein other than/in addition to the regulatory domain (as expanded previously in Results and Discussion). Available data also suggest that specific substitutions at 48 and 55 have similar functional effects in LacI and LLhP. We will next determine if these effects are consistently observed upon making substitutions in other members of the LacI/GalR family.

Residues 58 and 61 are clearly important for repressor function, since they are intolerant of most substitutions. The poor repressors of *lacO^I* could be trapped in a state that has low affinity for DNA (conformational change is precluded), or could have affinity altered so that repression can never occur from *lacO^I*. We observe residual allosteric response for many of the variants at position 61 (see Fig. 3), which indicates they must be capable of a conformational change. Therefore, our results are more consistent with a uniformly diminished affinity for *lacO^I*. In contrast, the 58 variants show no response to effector (see Fig. 3). Interestingly, the level of repression for the 58 variants is similar to values for the 61 mutants without hypoxanthine, but probably not as poor as repression by LLhP/Quad (see Fig. 3). Thus, the 58 variants may be capable of some degree of *lacO^I* binding/repression but cannot make the conformational change with the addition of effector. For both 58 and 61, the same substitutions appear to play different roles in the context of individual family members. A future goal will be to identify which regions of the regulatory domains mediate these effects.

Finally, members of the LacI/GalR family appear to have arisen from gene duplication with subsequent acquisition of new ligand specificities.⁷⁸ The latter process likely involved successive point mutations *en route* to the final linker sequences, which exhibit considerable diversity in the LacI/GalR family and in *E. coli* paralogues. A number of investigators are interested in the processes by which transcription factors evolve and whether this process is concurrent with evolution of different DNA-binding sites.^{79,80} We are extremely interested

to determine whether LLhP variants have different functional sensitivity to altered DNA, such as is seen when comparing intact LLhP and LacI (unpublished results). One possibility is that variant LLhP proteins that no longer recognize *lacO^I* have acquired specificity for completely different DNA binding sites; thus, such point mutations could dramatically alter the phenotype of an intact organism.

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Fig. 1. Protein structures. (A) The top panels show two views of the LacI dimer and the bottom two panels depict PurR. Figures were made with Chimera3 using the pdb files 1efa4 and 1wet.5 For each protein, the right panel is rotated 90° relative to that on the left in order to show differences in the linker regions. DNA is at the top of each structure (blue sticks) and is bound to the DNA-binding domain of each protein. Red highlights the N-linker (LacI 45–49); green, the hinge helix (LacI 50–58); and yellow, the C-linker (LacI 59–62). Homologous residues on LacI and PurR are colored the same, even though the hinge helix of PurR extends one residue farther than on LacI (solid arrows). Although the start of the N-linker and the beginning of the hinge helix may vary between the two proteins (dashed and dotted arrows, respectively), the

structural differences at these termini are less-pronounced. The regulatory domains are subsequent to the C-linker and shown in full for LacI in the upper left-hand structure. Anti-inducer and co-repressor are in black space-filling representation in the ligand-binding pockets between the two subdomains of the regulatory domains. **(B)** Cartoon of the LLhP chimeric protein. Ovals represent the DNA-binding domains, rectangles portray the linkers, and the large shapes depict the regulatory domains. Cyan indicates that the sequence is from LacI; lavender indicates that the sequence is from PurR. **(C)** Positions of linker residues that may convey unique functions to LacI/GalR family members,² shown on the structure of the LacI DNA-binding domain/linker. The N-linkers, hinge helices, and C-linkers are colored as in A.

Fig. 2.

Comparison of LacI and PurR linker-domain interfaces. Figures were generated with the program Resmap (<http://www.kumc.edu/biochemistry/swintkruse.html>),¹⁶ and edited with Microsoft PowerPoint. Columns of numbers represent residues in the protein sequences and are normalized to positions in LacI using the structure-based sequence alignment shown in Table I. Interface interactions between the linker residues and sites in other regions of the protein were identified using standard Resmap definitions. Interactions that are present in one protein and missing in the other are indicated with red and blue lines. Determination of “missing” interactions included an error of 0.5 Å: For example, if an interaction defined by a hydrogen bond in one structure (distance ≤ 3.5 Å) is missing in the second structure (distance ≥ 3.5 Å), the shortest distance between the two residues missing the interaction must also be >0.5 Å than the distance in the structure in which the interaction is present. PDB files used were 1efa (LacI bound to *lacO^{sym}*)⁴ and 1wet (PurR bound to *purf*).⁵ (A) Differences in linker

interactions between the two monomers within a dimer. Red lines depict interactions present only in LacI; blue lines represent interactions present only in PurR. **(B)** Differences in interactions between the linker residues and the DNA-binding and regulatory domains of the same monomer within a dimer. Dotted and solid lines are for LacI and PurR, respectively. The dashed line indicates a LacI interaction present on monomer B but not monomer A (it is asymmetric) that is also missing in PurR. **(C)** Cross-subunit interactions in the linker/linker and linker/regulatory domain interfaces that are conserved in LacI and PurR. **(D)** Same subunit interactions in the N-linker/DNA-binding domain and C-linker/regulatory domain interfaces that are conserved in LacI and PurR.

Fig. 3.

Results from liquid culture β -galactosidase assay of LLhP mutants. In these assays, low values of β -galactosidase activity correlate with increased repression. For panels showing data for LLhP variants with high repression (low β -galactosidase activity), average values are written above the bars of the graph. Striped bars represent values determined in the absence of co-repressor; dotted bars represent values determined from cells grown in the presence of adenine. Cells expressing LacI in the presence of IPTG (dotted bars) were assayed in parallel each day and serve as an internal calibration points: raw data for LacI + IPTG was set to 100 and data for LLhP variants were normalized relative to this value. Un-induced LacI had extremely strong repression (0.2 Miller units), probably due to DNA looping. Average values shown are from

3–6 independent assays (each with duplicate samples containing 1× and 2× cell culture volumes); error bars represent one standard deviation.

Fig. 4. Allosteric response of LLhP variants. Stippled bars represent the ratio of β -galactosidase units measured in the absence and presence of adenine. Error bars were propagated from the standard deviations shown in Figure 3.

Structure-Based Sequence Alignments for Regions of LacI and PurR^a

^aStructure-based sequence alignments of LacI and PurR were generated with CE/CL.⁷⁶ Triangles (▼) indicate the first and last positions of the linker used in LhP (top sequence). Different font styles and boxes are used to indicate the N-linker (dashed box), hinge helix (solid box), and C-linker (dotted box). Note that the hinge helix in PurR is one residue longer than the helix in LacI. Stars below the linker sequences indicate residues identified with the following three-part correlation²: (1) varied sequence in the LacI/GalR family, (2) structural differences between LacI and PurR, and (3) functional sensitivity to mutation in either LacI or PurR. Position 57 is known to be a determinant of DNA specificity in PurR⁷⁷ and thus is not considered in this study. Residues with light gray shading are extremely conserved linker sequences of the LacI/GalR family.^{30,78} Sequences for regions of the regulatory domains are also shown. Residues that make any interaction with the linker are indicated with striped backgrounds. If an interface residue is indicated for only one protein and also marked as common interaction on Figure 2(C) or (D), then the distance between the two residues of the homologous protein is within 0.5 Å. (See the legend to Fig. 2 for more explanation of this calculation.)

TABLE II

Primers Used in Construction of LLhP Variants

Primer name	Primer sequence
LLhPG58VFor	5'GCACAACAACCTGGCGGTCAAACAGAGCAAATCTATC3'
LLhPG58VRev	5'GATAGATTGCTCTGTTTGACCGCCAGTTGTTGTGC3'
LLhPS61TFor	5'GCACAACAACCTGGCGGGCAAACAGACCAAATCTATC3'
LLhPS61TRev	5'GATAGATTGGTCTGTTTGCCCGCCAGTTGTTGTGC3'
LLhPG58RDMFor	5'GCACAACAACCTGGCGNNNAAACAGAGCAAATCTATC3'
LLhPG58RDMRev	5'GATAGATTGCTCTGTTTNNCGCCAGTTGTTGTGC3'
LLhPS61RDMFor	5'GCACAACAACCTGGCGGGCAAACAGNNNAAATCTATC3'
LLhPS61RDMRev	5'GATAGATTNNNCTGTTTGCCCGCCAGTTGTTGTGC3'
LLhP148SFor	5'GAGCTCAATTACAGTCCCAACCGCGTGGCA3'
LLhP148SRev	5'TGCCACGCGGTTGGGACTGTAATTGAGCTC3'
LLhPQ55SFor	5'CGCGTGGCACAACTCACTGGCGGGCAAACAG3'
LLhPQ55SRev	5'CTGTTTGCCCGCCAGTGATTGTGCCACGCG3'
LLhPCSwapFor	5'GCACAACAACCTGGCGGTCAACCAACCAAATCTATC3'
LLhPCSwapRev	5'GATAGATTGGTGTGGTTGACCGCCAGTTGTTGTGC3'
LLhP148RDMFor	5'GAGCTCAATTACNNNCCCAACCGCGTGGCA3'
LLhP148RDMRev	5'TGCCACGCGGTTGGGNNNGTAATTGAGCTC3'
LLhPQ55RDMFor	5'CGCGTGGCACAAANNCTGGCGGGCAAACAG3'
LLhPQ55RDMRev	5'CTGTTTGCCCGCCAGNNNTTGTGCCACGCG3'
LLhPG58VS61TFor	5'GCACAACAACCTGGCGGTCAAACAGACCAAATCTATC3'
LLhPG58VS61TRev	5'GATAGATTGGTCTGTTTGACCGCCAGTTGTTGTGC3'
LLhPQ55SG58VFor	5'CGCGTGGCACAACTCACTGGCGGTCAAACAG3'
LLhPQ55SG58VRev	5'CTGTTTGACCGCCAGTGATTGTGCCACGCG3'

TABLE III

Phenotypes of LLhP Linker Variants^a

Mutation ^b	(-)Adenine ^c	(+)Adenine ^d	Phenotype in LacI ^e	C3 propensity ^f
LLhP	White	White		4
I48G	Blue	Blue	—	
I48H	Light blue	Light blue	—+	
I48L	Blue	Blue	—+	
I48R	Blue	Blue	—+	
⊗I48S	White	White ^g	—	
I48T	White	Light blue		
I48V	White	White		
Q55A	Light blue	Light blue ^h	+	3
Q55G	Blue	Blue	—	19
Q55I	White	White		11
Q55P	Blue	Blue	—	20
⊗Q55S	Blue	Blue	—+h	17
Q55T	White	White		16
Q55V	White	White		15
Q55Y	Blue	Blue	+—	12
G58A	Blue	Blue	—+	
G58I	Blue	Blue		
G58L	Blue	Blue	—	
G58P	Blue	Light blue	—	
G58R	Blue	Blue	+	
G58S	Blue	Blue	—+	
G58T	Blue	Light blue		
⊗G58V	Blue	Blue		
G58Y	Blue	Blue	—	
S61C	Light blue	Light blue	+	
S61D	Blue	Blue		
S61H	Blue	Blue	ws	
S61L	Blue	Light blue	s ⁱ	
S61M	Light blue	Light blue		
S61Q	Blue	Light blue	+	
S61R	Blue	Blue	+	
⊗S61T	Blue	Blue		
S61V	Blue	Blue		
S61W	Blue	Blue		
⊗G58V, S61T	Blue	Blue		
⊗Q55S, G58V, S61T	Blue	Blue		
⊗LLhP/Quad (I48S, Q55S, G58V, S61T)	Blue	Blue		
⊗LLhP/Cswap (G58V, K59N, Q60H, S61T)	Blue	Blue		

^a White colonies indicate repression of the reporter genes; blue colonies indicate no repression is occurring, and β-galactosidase reporter protein is expressed.

^b Mutations that exchange residues between PurR and LacI are indicated with ⊗. Other variants were generated with random mutagenesis.

^c In the absence of co-repressor, both PurR and LLhP have lowered affinity for operator DNA.

^d Adenine is metabolized to co-repressor hypoxanthine. When co-repressor binds PurR or LLhP, the protein has higher affinity for operator DNA (unpublished results for LLhP).

^e Results are from LacI mutagenesis studies in the Miller lab measured using a liquid culture, β-galactosidase assay.²⁸ “+” indicates that the LacI variant can repress and responds to inducer IPTG. “+—” and “—+” indicate diminished repression, probably similar to the “light blue” phenotype of the current plate assay. “s” indicates a LacI variant that can repress transcription but is not inducible; “h” indicates a heat sensitive mutation.

^f Rank order of helical propensities at the C3 position, with lower number corresponding to higher propensities; Q ranks fourth.⁵⁸

^g Although I48S is white in the plate assay, the liquid culture assay demonstrates diminished repression for this variant (Figure 3), consistent with the LacI I[−] result.

^h The apparent mismatch for Q55A in LLhP and LacI may result from the tighter repression of looped, tetrameric LacI.

ⁱ This substitution causes tighter DNA binding in LacI, including increased non-specific affinity.⁶⁰