Amino acid alterations were designed at the C terminus of the hinge segment (amino acids 51–59) that links two functional domains within lactose repressor protein (LacI). Gly was introduced between Gly58 and Lys60 to generate Gly58+1; Gln60 was changed to Gly or Pro, and up to three additional glycines were inserted following Gln60 → Gly. All mutant proteins exhibited purification behavior, CD spectra, assembly state, and inducer binding properties similar to wild-type LacI and only small differences in trypsin proteolysis patterns. In contrast, significant differences were observed in DNA binding properties. Gly58+1 exhibited a decrease of ~100-fold in affinity for O1 operator, and sequential Gly insertion C-terminal to Gln60 → Gly resulted in progressively decreased affinity for O1 operator, approaching nonspecific levels for insertion of ≥2 glycines. Where sufficient affinity for O1 operator existed, decreased binding to O1 in the presence of inducer indicated no disruption in the allosteric response for these proteins. Collectively, these results indicate that flexibility and/or spacing between the core and N-terminal domains did not significantly affect folding or assembly, but these alterations in the hinge domain profoundly altered affinity of the lactose repressor protein for its wild-type target sequence.

The lac repressor (LacI) negatively regulates lac mRNA synthesis by binding with high affinity to its target operator site and thereby precluding transcription by RNA polymerase (1–6). In response to the presence of inducer sugars, LacI undergoes a conformational change that results in diminished affinity for operator sequences without effect on nonspecific DNA binding (1, 2, 7). LacI is a protein of 150,000 Da composed of four identical subunits assembled as a dimer of dimers (see Fig. 1) (7–10). The N-terminal 60 amino acids in each monomer form a helix-turn-helix motif that directly contacts the operator DNA (7, 11–17). When isolated, this region exhibits site-specific DNA binding, albeit with significantly lower affinity than the intact protein (11, 18, 19). The LacI core domain consists of residues 60–360, which encompass the inducer-binding site and the assembly determinants for the protein (7, 10, 20–30).

The binding of inducer to the core domain results in disruption of specific DNA binding (1, 2), apparently because of structural shifts transmitted to the N termini that alter their relative orientation and thereby preclude complementarity with the operator sequence (7, 26, 27). Assembly to tetramer is mediated by two separate regions: (a) a monomer-monomer interface that is generated by contacts throughout the core domain primary sequence and (b) a dimer-dimer interface formed by a short segment at the C terminus of the protein (amino acids 340–360) that assembles via a leucine heptad repeat sequence into a 4-helical anti-parallel coiled-coil structure (7, 10, 21–30).

The N-terminal DNA-binding domain has been studied extensively by NMR spectroscopy, an approach necessitated by the inability to discern electron density for the N-terminal domain in the crystal form without operator present (7, 10, 13–17, 31, 32). This DNA-binding domain has considerable structure when isolated from the remainder of the protein, and this region remains remarkably mobile even when attached to the larger and more rigid core domain (33). The segment that covalently connects the N-terminal DNA-binding domain to the tetrameric core protein is the hinge formed by amino acids 51–59, and flexibility in this region may contribute to the observed N-terminal freedom of motion. The proteolytic susceptibility of this region was ascribed to the absence of stable secondary structure, consistent with the motional flexibility of the attached N-terminal DNA-binding domain (18, 19, 33–36). The large ΔCp observed for LacI-operator binding has been attributed to a local folding transition in the protein that buries apolar residues, a process potentially corresponding to hinge helix formation (37). The folded hinge region in the crystal structure of LacI bound to a symmetric operator DNA appeared to confirm this interpretation (7). In addition, NMR analysis demonstrated helix formation by the hinge region in isolated N-terminal domains when complexed with full-length operator but not in the free state (31). Similarly, the hinge helix of the LacI homolog, PurR, occurs only in the presence of the cognate operator sequence (38, 39). In contrast to LacI, folding of the PurR hinge helix is not observed for the isolated PurR N-terminal DNA-binding domain, even in the presence of specific DNA sequences (40). Whether this difference derives from the distinct effects of effector ligand (corepressor versus inducer) or other sources is not yet established. DNA binding in other proteins has been shown to involve α-helix formation from an unfolded segment in conjunction with complex formation (41–43). Thus, protein recognition of site-specific DNA appears in many cases not to be an interaction between rigid bodies but rather involves coupled changes in the structure of the protein (37) and potentially in the DNA (44).

The significance of the small hinge region that links the N terminus and core domains to overall LacI function has motivated a more detailed analysis of this flexible segment. Extensive genetic studies on the LacI gene have demonstrated that multiple substitutions within the hinge diminish operator binding (26, 27, 45, 46). Based on proteolytic, crystallographic, and NMR studies, the hinge region assumes structure in the presence of DNA, and this connecting segment may therefore
be important for the allosteric transition that effects communication of operator and inducer binding (7, 26, 27, 33, 47, 48). To explore the contribution of the hinge segment to DNA recognition and to allosteric communication within LacI, we have altered flexibility and/or spacing within this region. Glycinines are found at turns and bends of a folded protein structure and are presumed to be flexible at least in part because of the absence of a side chain (49, 50). We have therefore made a series of mutations in the hinge region that either alter a single amino acid side chain or introduce one or more additional glycines into the amino acid sequence at selected sites (see Table I and Fig. 1B). The results obtained with these mutant proteins indicate that high affinity DNA binding is closely coupled with the sequence of the hinge region and that precise orientation appears important for optimal interaction of this regulatory protein with its cognate operator sequence.

**MATERIALS AND METHODS**

**Bacterial Cells and Growth**—Bacterial strain *Escherichia coli* DH5-α (F′endA1hisD17([r639 m59] supE44 thi−lrecAl gyrA[Na+] retAl ΔlacI74-argF'), U169deoR) was used for DNA purification. Proteins were expressed using *E. coli* BL26 cells (BL26Blue cells from Newagen (Madison, WI), which are ompF, β-lactamase, and gal dcm lac[F′proABlac+3 Δ456:3 (θ)lac]) and are presumed to be flexible at least in part because of the absence of a side chain (49, 50). Cells were grown in 2 Y medium (16 g/liter tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl, pH 7.4) for liquid culture. LB medium (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl, pH 7.4) with 15 g/liter agar was used for plates. Selection antibiotics were present at 50 μg/ml for a final concentration.

**Site-specific Mutagenesis**—Mutations in the lac repressor were generated on plasmid pJCl (25) using the Chameleon mutagenesis protocol from Stratagene. The mutagenic and selection oligonucleotides (Great American Gene Co. or Genosys) were used at least 100-fold concentration over template. The mutagenesis mixture was transformed into *E. coli* XLmuts cells (Stratagene) or *E. coli* mutS cells (Promega) that were subsequently grown overnight in 5 ml of 2 Y medium. Plasmid DNA was purified and examined by restriction digestion. Following retransformation into *E. coli* DH5-α cells, individual colonies were selected for growth and purification of plasmid DNA. Plasmid DNAs from individual colonies that digested with the selection enzyme were sequenced to determine whether they also carried the mutation. Plasmids carrying the appropriate mutation were sequenced in their entirety to confirm that the designed change was the only alteration in sequence present.

**Protein Purification**—Purification of lactose repressors followed the previously described procedure for wild-type protein (51, 52), with some modifications. The cell lysis supernatant was precipitated with 40% ammonium sulfate and allowed to incubate at 4 °C for at least 30 min. The pellet was resuspended in buffer containing 0.09 M potassium phosphate buffer, pH 7.5, 5% glucose, 0.3 mM dithiothreitol. This solution was then dialyzed overnight at 4 °C against the same buffer. The dialyzed fraction containing the lac repressor protein was centrifuged at 9000 rpm for 30 min to remove any precipitate before loading onto a phosphocellulose column equilibrated with 0.09 M potassium phosphate, pH 7.5. After loading the protein suspension, the column was washed with the same buffer, followed by 0.12 M potassium phosphate buffer, and then eluted with a gradient from 0.12 to 0.3 M potassium phosphate. Fractions containing the lac repressor protein were collected, and concentrations were determined using absorbance at 280 nm, using wild-type lac repressor as a standard. Throughout purification and isolation, the protein activity was detected by the [14C]IPTG assay described by Wycuff and Matthews (26) and was confirmed by circular dichroism using an Aviv 62DS spectropolarimeter with a 2-mm-path length quartz cuvette. Protein concentration was 4 mg/ml for a 30% protein solution.

**TABLE I**

| LacI hinge region* | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 |
|--------------------|----|----|----|----|----|----|----|----|----|----|----|----|
| Mutants            | 58 | 59 | 60 | 61 |
| Wild type          | Gly | Lys | Gly | Ser |
| Gly58→1            | Gly | GLY | Lys | GLY | Gln | Ser |
| Gln59→Gly          | Gly | Lys | GLY | GLY | GLY | GLY | Ser |
| Gly59→2            | Gly | Lys | GLY | GLY | GLY | GLY | Ser |
| Gln59→Pro          | Gly | Lys | GLY | GLY | GLY | GLY | Pro | Ser |

* Amino acids in capital letters represent the mutations and/or insertions engineered within the hinge region.

1 D. Wycuff and K. S. Matthews, submitted for publication.

2 The abbreviation used is: IPTG, isopropyl-β-D-thiogalactoside.
buffer, pH 7.6. Elution volume was determined by monitoring absorbance at both 230 and 280 nm. All mutant proteins eluted at the same volume as determined for wild-type lac repressor.

**Proteolysis of Protein**—Individual proteins were diluted to 0.3 mg/ml in 1 M Tris-HCl, pH 7.6, 30% glycerol at room temperature. Trypsin (1 mg/ml in 1 mM HCl) was added to obtain a 2% (w/w) final solution (18). An aliquot of undigested protein was removed prior to trypsin addition. Digestion was carried out at room temperature, samples were taken at designated time points, and further digestion was inhibited with addition of 4% (w/v) phenylmethylsulfonyl fluoride (4 mg/ml in 100% EtOH). Samples were run on 10% SDS-polyacrylamide gel electrophoresis to visualize digestion patterns.

**Repressor-Inducer Binding**—Binding of inducer was assessed by monitoring the change in fluorescence emission intensity (54). The protein concentration for wild-type lac repressor and all mutants was $1.5 \times 10^{-7} M$ monomer. The final inducer concentrations ranged from $6.7 \times 10^{-8} M$ to $1.8 \times 10^{-7} M$ for assays done at pH 7.6 and from $1.3 \times 10^{-7} M$ to $4.4 \times 10^{-8} M$ for assays done at pH 9.2. Proteins were diluted into 0.01 M Tris-HCl, 0.15 M KCl buffer at the specified pH. The fluorescence emission was monitored on an SLM-Aminco 8100 spectrofluorometer using a 340-nm cut-off filter (O-52) from Corning with an excitation wavelength of 285 nm (55, 66). Fluorescence intensity correction factors for dilution and photobleaching were generated using an identical titration with buffer solution rather than IPTG. Data were analyzed by nonlinear least squares analysis to fit to the binding equation,

$$R = Y_m \times \frac{[\text{IPTG}]^n}{K_d [\text{IPTG}]^n + [\text{IPTG}]^n}$$  
(Eq. 1)

where $R$ is fractional saturation (change in fluorescence signal at a particular IPTG concentration divided by the maximum change in fluorescence signal), $Y_m$ is a factor that allows the maximum value of $R$ to float, $K_d$ is the apparent equilibrium dissociation constant, and $n$ is the Hill coefficient.

IPTG titrations for selected proteins were also performed in buffer at pH 7.6 in the presence of near saturating amounts of specific operator sequences to assess the effect on inducer affinity. Protein concentrations were $1.5 \times 10^{-7} M$ monomer. The final IPTG concentrations were varied. The operator concentration was $5.0 \times 10^{-7} M$.

**Repressor-Operator Binding**—A 40-base pair double-stranded DNA corresponding to the wild-type O1 operator (5'-TGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGG-3') was used to measure operator affinity for the mutant proteins. The oligonucleotides (Great American Gene Co.) were purified by polyacrylamide gel electrophoresis and eluted from the gel by incubation overnight in TE buffer at 37 °C with gentle agitation. The top and bottom strands were hybridized in annealing buffer (8 mM Tris-HCl, pH 7.5, 4 mM MgCl2, 10 mM NaCl), and this material was labeled at the 5' end with $32P$ by polynucleotide kinase reaction. A Nick column (Amersham Pharmacia Biotech) was used to purify the labeled 40-base pair operator from the free nucleotides after the reaction was completed.

Operator binding was assayed using the nitrocellulose filter binding assay (51, 56) modified for use in a 96-well dot blot apparatus (57). The assay was performed at room temperature in buffer containing 0.01 M Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.15 M KCl, 5% dimethyl sulfoxide. The proteins were diluted into this buffer with the addition of 50 µg/ml bovine serum albumin. The concentration of labeled operator used in the assay was $1.3 \times 10^{-13} M$ for tight-binding mutants, $1.0 \times 10^{-12} M$ for normal binding, and $1.0 \times 10^{-11} M$ for low affinity binding. The concentration of protein ranged from $1.0 \times 10^{-13} M$ to $1.0 \times 10^{-6} M$ tetramer depending upon the affinity of the repressor for operator. The amount of

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**FIG. 2.** Circular dichroism spectra for wild-type and mutant lac repressors. The circular dichroism spectra were measured as described under "Materials and Methods." The protein concentration was 0.15 mg/ml in 0.12 M potassium phosphate buffer, pH 7.6. Spectra are shown for wild-type LacI (●), Gly58→1 (○), Gly60→Gly (□), Gln60→Pro (▼), Gly60→1 (△), Gly60→2 (▲), and Gly60→3 (●).
Protein was mixed with labeled operator (1 \times 10^{-13} M) and protein concentrations were determined from the repressor-operator binding assays to generate ~80% complex formation. The concentration of IPTG varied from 2 \times 10^{-7} M to 2 \times 10^{-4} M. Once inducer was added, the reaction mixture was allowed to incubate at room temperature for 20–30 min and then was filtered onto nitrocellulose. The amount of 32P-labeled operator bound at the indicated pH was measured by nitrocellulose filter binding assay. Each value was determined by fitting at least three curves simultaneously to Equation 1. Error values were generated by Igor Pro and represent the standard deviation from the fitted curve.

RESULTS AND DISCUSSION

Rationale for Mutagenesis and Production of Mutant Proteins—Genetic studies suggested that Gly58-Lys59 at the C-terminus of the LacI hinge helix may be important for DNA binding (45, 46), and other analyses have expanded this view (58, 59). The ends of an α-helix are important to stabilize the structure and “cap” the end of the helix (60–62), and glycines are commonly found in the sequence of C-terminal caps (63, 64).

Table II

| Protein                | Operator (O1) $K_d$ | Inducer (IPTG) $K_d$ |
|------------------------|--------------------|----------------------|
|                        | $M \times 10^6$    | $M \times 10^6$      |
| Wild type              | 1.2 ± 0.2          | >10000 1.7 ± 0.2 23 ± 2 |
| Gly58-1                | 150 ± 50           | >10000 1.9 ± 0.1 17 ± 2 |
| Gly58-1 Gly            | 4.5 ± 0.5          | >10000 1.8 ± 0.2 18 ± 1 |
| Gly58-1-2              | 500 ± 200          | >10000 1.5 ± 0.2 21 ± 1 |
| Gly60-1-3              | >10000             | >10000 1.9 ± 0.1 21 ± 2 |
| Gly60-1 Pro            | 7.4 ± 1.3          | >10000 2.0 ± 0.1 32 ± 1 |

$^a$ The apparent dissociation constant for 40-base pair operator DNA ($1 \times 10^{-12} M$) was measured by nitrocellulose filter binding assay. Each value was determined by fitting at least three curves simultaneously to Equation 2. Error values were generated by Igor Pro and represent the standard deviation from the fitted curve.

$^b$ The relative affinity of the protein for IPTG at the indicated pH was measured by fluorescence methods. Each value was generated by fitting at least three curves simultaneously to Equation 1. Error values were generated by Igor Pro and represent the standard deviation from the fitted curve.

$^c$ The proteins were also assayed with a nonspecific 40-base pair operator (“Materials and Methods”), and the binding isotherms were similar to those in the presence of inducer, generating $K_d$ values greater than $1 \times 10^{-7} M$.

![Fig. 3. Inducer binding curves for mutant proteins.](image-url) Fluorescence titrations were performed on an SLM 8100 spectrofluorometer as described under “Materials and Methods.” The concentration of protein was $1.5 \times 10^{-7} M$ monomer in 0.01 M Tris-HCl, 0.15 M KCl at the indicated pH. Assays were performed at pH 7.6 (●) and pH 9.2 (○). The curves were generated by simultaneously fitting all the data to Equation 1. Three replicates were included for each assay condition.
Moreover, lysines are sometimes found at the C terminus of an α-helix, because the positive charge can interact with the helix dipole and the long side chain can fold back to stabilize the conformation via hydrophobic interactions (64, 65). Inspection of the hinge helix in LacI suggests that Gly58 may be involved in such a cap and that Lys59 may play a role in stabilizing the hinge helix (Table I). Thus, insertion of additional glycines between these two amino acids might disrupt the conformation of the entire region. For the first mutation, an oligonucleotide was designed to incorporate an extra glycine that was C-terminal to Gly58 (Gly58-Pro), separating Gly58-Lys59. Position 60, at the C-terminal end of the hinge region, is tolerant to different amino acids based on phenotype from genetic studies and presumably therefore does not have a structural role in the hinge helix or in LacI function (26, 27, 45, 46). This position was therefore chosen as an alternative site for glycine substitution and insertion. In a second series, Gln60 was mutated to glycine (Gln60 → Gly), and then, sequentially, glycines were inserted to generate a second series of glycine insertions (Table I). As an alternate substitution, Gln60 → Pro was used to assess the effect of minimized conformational flexibility on function. Proline constrains the peptide backbone, thus making the polypeptide chain locally less flexible (49).

Following double-stranded mutagenesis to introduce designed changes in DNA sequence, the plasmids for each mutation were sequenced throughout the lacI gene to verify the desired change and to confirm the absence of other DNA sequence alterations. These constructs were then transformed into E. coli BL26 cells for protein expression. The proteins were all expressed at high levels and were purified readily according to the protocol for the wild-type repressor protein (see Fig. 4 for gel electrophoresis results).

Secondary Structure and Assembly of Mutant Proteins—The purification properties of the mutant repressors suggested that they possess structure similar to the wild-type protein. To confirm this conclusion, circular dichroism measurements were used to demonstrate that the secondary structure content for all of the mutant proteins was similar to wild-type LacI (Fig. 2). Furthermore, all of the mutant proteins eluted at a volume similar to wild-type LacI from molecular sieving columns (data not shown), indicating that the oligomeric structure was also wild-type in nature. Thus, none of this series of mutant proteins exhibited folding or assembly characteristics distinct from wild-type lacI repressor.

Inducer Binding Properties—Similarly, inducer binding at pH 7.6 was observed to be very similar to wild-type LacI for all of the mutant proteins in this series (Table II and Fig. 3). Thus, the tertiary fold of the monomer required for contacting the sugar within its binding site was maintained in all of the mutant proteins. At pH 9.2, wild-type LacI exhibits a lower affinity for IPTG and shows cooperativity in binding under these conditions (25, 67). This pH-dependent behavior mimics the response seen when IPTG binding is assayed in the presence of saturating amounts of operator and relies on the integrity of the monomer-monomer interface (25, 55, 67). When assayed at elevated pH, all the mutant proteins demonstrated similar binding affinities to wild-type lacI repressor. Thus, communication between the subunits within a dimer, reflected in the pH-dependent behavior for inducer binding (25), is not influenced by the hinge segment mutations. These data demonstrate that the core domain, which must fold effectively to form the inducer-binding site, is intact in the mutant proteins and that the monomer-monomer subunit interface necessary for cooperativity also is unaffected by these mutations.

Proteolysis of Mutant Proteins—Mild proteolysis of wild-type LacI has been shown to be very specific under certain solution conditions and proceeds to only a limited extent (18, 34–36). This phenomenon presumably results from folding of a specific region to occlude an otherwise susceptible bond that consequently becomes resistant to cleavage (68). With mild proteolytic digestion of lacI repressor, the DNA-binding domain is separated from the inducer-binding domain with loss of high affinity operator recognition (18, 19, 34, 69). Trypsin cleavage was found to occur following amino acids Arg51 and Lys59, which flank the hinge sequence (18, 19, 35, 36). The mutant proteins generated in this study were assessed by mild trypsin digestion to determine whether the amino acid changes or additional glycines rendered the hinge region altered in protease susceptibility compared with wild-type LacI (Fig. 4). From these experiments, the insertion mutants exhibited approximately the same digestion products as wild-type lacI repressor. The wild-type protein was 50% digested within 30 min. Gly60 → Gly, Gly60+1, and Gly58+1 had comparable digestion patterns to wild-type protein. Gly60+2 and Gly60+3 demonstrated slightly accelerated digestion, presumably a result of the additional flexibility and/or accessibility of the target region. Gly60 → Pro was digested by trypsin at a decreased rate compared with wild-type protein and to a larger product, a result derived at least in part from decreased susceptibility of the scissile peptide bond when a proline follows a lysine (70). The small population of lower molecular weight bands may be a result of C-terminal digestion (18, 36). Based on these results, the hinge region does not appear to be significantly altered in
its solvent/protease exposure by the amino acid changes introduced in the hinge region.

**Operator DNA Binding Properties**—This series of proteins was assayed using the wild-type primary operator sequence, \( \text{O}^1 \) (Table II and Fig. 5). For the \( \text{Gly}^{58} \rightarrow \text{Gly} \) protein, significant loss of binding affinity was observed, with a \( K_d \) of \( 1.5 \times 10^{-9} \text{M} \) for \( \text{O}^1 \) compared with \( 1.2 \times 10^{-11} \text{M} \) for wild-type LacI. In sharp contrast, the substitution mutant, \( \text{Gln}^{60} \rightarrow \text{Gly} \), had a higher affinity for \( \text{O}^1 \) compared with wild-type protein, with a \( K_d \) of \( 4 \times 10^{-12} \text{M} \). With sequential glycine insertion following \( \text{Gly}^{60} \), the affinity for \( \text{O}^1 \) decreased until the \( K_d \) approached that for nonspecific DNA binding, \( 4 \times 10^{-13} \text{M} \) (70). \( \text{Gln}^{60} \rightarrow \text{Pro} \) exhibited only a 6-fold reduced affinity for \( \text{O}^1 \) compared with wild-type LacI. Although increasing flexibility in \( \text{Gln}^{60} \rightarrow \text{Gly} \) increased affinity, increasing the “rigidity” of this region in \( \text{Gln}^{60} \rightarrow \text{Gly} \) appeared to impair recognition of the operator sequence but only moderately. Wild-type and mutant proteins were assayed with a nonspecific DNA sequence, and the equilibrium dissociation constant was \( 10^{-12} \text{M} \) (Table II, footnote c) (71). The binding to nonspecific DNA for the mutant proteins appeared unaltered compared with the wild-type protein.

The diminution in DNA binding observed in \( \text{Gly}^{58}+1 \) and in \( \text{Gly}^{60}+1 \), \( \text{Gly}^{60}+2 \), and \( \text{Gly}^{60}+3 \) may derive from several effects of these alterations: (a) The entropic cost of binding anticipated to accompany the increased flexibility derived from Gly substitutions may exact an increasing cost in the protein-operator interaction as the number of Gly residues increases. (b) An effect on hinge helix folding by the substitutions may compromise affinity. (c) Misalignment of the operator half-sites with the two N-terminal DNA-binding domains within a dimer may be elicited by alterations in N-terminal-core spacing with the consequence of diminished DNA binding affinity. (d) Essential interactions between the N-terminal and core domains may be disrupted by these hinge alterations. Each of these factors may contribute differentially to the observed effects on each mutant protein. Distinguishing these effects will require further exploration of these mutants.

**Release of Operator by IPTG**—The importance of the hinge helix in transmitting the induction signal between the sugar-binding site in the core domain and the N-terminal DNA-binding domain has been suggested (7, 26, 27, 34, 47, 48). To explore the effect of inducer on operator binding for this series of mutants, \( \text{O}^1 \) affinity in the presence of IPTG was determined (Fig. 5). Operator binding was diminished for all the mutant proteins in the presence of saturating amounts of inducer; a difference was apparent even for \( \text{Gly}^{60}+3 \), for which binding approached nonspecific levels. This result demonstrated directly that the allosteric response was not disrupted in these mutant proteins, even when DNA binding was severely diminished. \( \text{Gln}^{60} \rightarrow \text{Pro} \), with presumably increased rigidity in this region, also demonstrated a significant decrease in affinity in the presence of saturating amounts of inducer, suggesting that constraining this region inhibits optimal operator recognition but does not affect the allosteric response.

To quantitate the release of DNA by the presence of IPTG, an additional assay was employed to examine those mutants with...
Effect of Flexibility and Spacing in LacI Hinge Region

To explore the effect of flexibility and spacing in the hinge region on DNA binding, we have measured the binding of inducer in the presence of O1 operator DNA. The purification parameters, inducer binding properties, molecular mass, folding as assessed by circular dichroism, and sensitivity to protease were all comparable with those of the wild-type protein. Thus, mutation in the hinge region does not alter the helix, and alteration of the sequence and/or spacing at the C terminus of the hinge, perhaps by interfering with a helix cap, may impede formation of the high affinity DNA-binding site; (Gly58→Pro decreased O1 operator DNA binding by only 6-fold, and addition of a Gly to Gly58→Pro (Gly58→Pro) decreased operator binding compared with wild-type LacI by <4-fold. These changes in the hinge sequence therefore altered binding to O1 operator only minimally. In contrast, addition of further Gly residues to Gly58→Pro disrupted DNA binding significantly, with a 104 difference between Gly58→Pro and Gly58→Pro binding affinity. Interestingly, the allosteric response to inducer was unaffected in this series of mutant proteins; the addition of inducer diminished binding for all of the mutants.

The only property affected significantly by these mutations in the hinge domain of the protein was binding to O1 operator DNA. The purification parameters, inducer binding properties, molecular mass, folding as assessed by circular dichroism, and sensitivity to protease were all comparable with those of the wild-type protein. Thus, mutation in the hinge region does not exert significant influence outside this domain, in contrast to other mutations that have long range effects on structure and function (28, 29, 30, 72). The exclusive effect of the mutations examined in the hinge segment is on operator DNA binding. Furthermore, all the mutant proteins exhibit allosteric response to inducer binding, and, consistent with thermodynamic principles, the extent to which the presence of O1 operator DNA diminished inducer binding correlated directly with the allosteric effect of IPTG on operator binding.

The specific mechanism by which introduction of additional flexibility/spacing in the hinge region reduces specific operator recognition, as observed in particular for Gly58→1, Gly58→2, and Gly58→3 cannot be unequivocally deduced from the current information. At least four potential contributions to disruption of binding can be identified. Increased flexibility in the region linking the core and DNA-binding domains may (a) increase the entropic cost of binding and thereby diminish affinity; (b) alter the spacing of the N-terminal DNA-binding domains with respect to one another within a dimer and therefore preclude formation of the high affinity DNA-binding site; (c) affect fold-
ing of the hinge helix required for minor groove insertion; or (d) affect the ability of the N-terminal DNA-binding domain to interact with the core domain, an arrangement potentially required for alignment of the N termini and consequently DNA binding. The hinge alterations generated in LacI could influence any of these factors.

At least one of these factors appears important for binding of PurR to its operator DNA. A recent demonstration that the binding of the LacI homolog, PurR, to operator DNA can be disrupted by mutation at core residue Arg115, which appears to form a hydrogen bond with the backbone of Ser46 in the partner subunit N terminus, suggested that N-terminal-core interactions may be important in PurR DNA binding and/or allostery (73). Of note in this context is the effect of substitution at Arg118, the homologous residue in LacI; phenotypic measurements indicate that substitution of this residue results in protein unable to bind effectively to operator in vivo (26, 27, 45, 46). Whether alterations in the hinge region affect core-N-terminal interactions or alter the three-dimensional arrangement of the N-terminal, hinge, and core domains will require crystallographic structural analysis of these mutant proteins. What is evident, however, from comparing the data for Gly60+1 and Gly60+2 or for wild type and Gly58+1 is that lengthening the polypeptide backbone that connects the N terminus and core domain in lactose repressor protein can have a profound effect on O1 operator DNA binding parameters. Despite the significant changes in O1 operator affinity, insertion of additional amino acids did not disrupt the allosteric response. From the crystallographic structures (7), inducer binding elicits a conformational change that shifts amino acid 62 ~3.5 Å away from its partner within the dimer. This structural shift presumably disrupts hinge helix interaction with DNA and may preclude hinge helix folding. The separation of N-terminal DNA-binding domains and potential loss of the hinge helix would abolish high affinity specific binding. Insertion of glycine residues adds additional “distance” between the N-terminal DNA binding and core inducer-binding domains with the consequence of significant loss in O1 binding affinity but maintenance of inducibility. Thermodynamic studies are underway to assess the entropic contribution to binding, and these measurements may indirectly assess effects on hinge helix folding based on derived \( \Delta C_p \) values.

The hinge region of the LacI protein is key to the DNA binding activity of the tetramer, and recognition of the primary operator sequence is influenced significantly by specific alterations in this segment that increase flexibility and/or extend the polypeptide backbone. The ability to specifically modify DNA binding alone, while maintaining the structure and other binding properties of the protein, provides an opportunity to explore the role of DNA sequence and structure in a varied background of LacI proteins. Of particular interest in future studies will be examination of the effect of operator sequences with altered spacing between half-sites on DNA binding parameters for these proteins to determine whether insertion of Gly residues alters the spacing of half-sites required for optimal binding.
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