Substitutions at Histidine 74 and Aspartate 278 Alter Ligand Binding and Allostery in Lactose Repressor Protein†

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ABSTRACT: In the inducer-bound structure of the *lac* repressor protein, the side chains of H74 and D278 are positioned to form an ion pair between monomers that appears to be disrupted upon operator binding (Lewis, M., Chang, G., Horton, N. C., Kercher, M. A., Pace, H. C., Schumacher, M. A., Brennan, R. G., and Lu, P. (1996) *Science 271*, 1247−1254). A series of single substitutions at H74 and D278 and a double mutant, H74D−D278H, were generated to determine the influence of this interaction on ligand binding and allostery in *lac* repressor. Introduction of apolar amino acids at H74 resulted in distinct effects on ligand binding. Alanine and leucine substitutions decreased operator binding, while tryptophan and phenylalanine increased affinity for operator DNA. Introduction of a negatively charged residue at position 74 in H74D had minimal effects, and "inverting" the side chains in H74D/D278H did not significantly alter inducer or operator binding at neutral pH. In contrast, all substitutions of D278 increased affinity for operator DNA and diminished inducer binding. These observations can be interpreted in the context of the Monod−Wyman−Changeux model. If a salt bridge were essential for stabilizing or destabilizing the inducer-bound conformation, a mutation at either residue that interrupts this interaction should have a similar effect on allostery. Because the type and degree of alteration in ligand binding properties depended on the nature of the substitution at these residues, the individual roles played by H74 and D278 in *lac* repressor allostery appear more important than their direct contact across the monomer−monomer interface.

The transcription of the genes required for lactose transport and metabolism is regulated by the *lac* repressor protein in a process that involves its allosteric interaction with inducer sugars and specific operator DNA sites (*1*). In response to binding inducer sugars, the affinity of *lac* repressor protein for specific operator DNA sequences decreases ∼4 orders of magnitude (*2*−*5*). This alteration in operator DNA affinity results in the transcription of the *lac* operon genes by RNA polymerase (*1*). Conversely, occupation of operator sites reduces the affinity of the protein for inducer sugars and thereby determines the threshold level of inducer sugar necessary for induction (*5*−*7*). The regions of the protein involved in binding to these different ligands have been determined by genetic and structural studies (*8*−*14*).

The repressor consists of three domains: a helix-turn-helix DNA binding region (amino acids ∼1−50) (*14*−*18*), a bilobate core domain (amino acids ∼62−340, displaying homology to periplasmic binding proteins) with the sugar binding site located between the N- and C-subdomains (*8*−*14*, *19*−*22*), and a leucine heptad repeat domain (amino acids 340−360) that forms the dimer−dimer interface (*23*−*25*). In addition to this dimer−dimer assembly motif, tetramer assembly requires a monomer−monomer interface involving numerous contacts between two of the core domain monomers (*10*, *13*, *14*, *19*, *26*). Mutation of residues along the monomer−monomer interface can interfere with communication between the two ligand binding sites (*26*−*28*, *51*).

Although the detailed allosteric mechanism for induction is not known, a model has been proposed on the basis of the crystal structures of the different liganded states of this repressor protein (*14*, *29*). A comparison of these crystal structures of *lac* repressor identified the regions that are reoriented upon ligand binding (*14*). On the basis of this analysis, *lac* repressor conformational changes arise largely from the movement of monomers relative to each other and not on large interdomain alterations (*14*). The structural basis for the effect of inducer on operator binding may involve a "hinge" helix located between the N-terminal helix-turn-helix and core domains (*14*, *29*). In the presence of specific contacts with operator, this hinge helix makes important minor groove contacts (*30*). When inducer sugar binds, the N-subdomains of the core monomer−monomer interface rotate, and this movement may preclude both minor and major groove protein−operator contacts. While the rotation of the monomers relative to each other is ∼10° in the N-subdomain of the core, the C-subdomain remains in a fixed orientation (*14*). The reorientation of the N-subdomain upon ligand binding to the core is similar to that seen in structures of purine repressor, PurR[1] (*31*, *32*). Three pairs of potential electrostatic bonds were detected within the *lac* repressor monomer−monomer interface in the inducer-bound struc-

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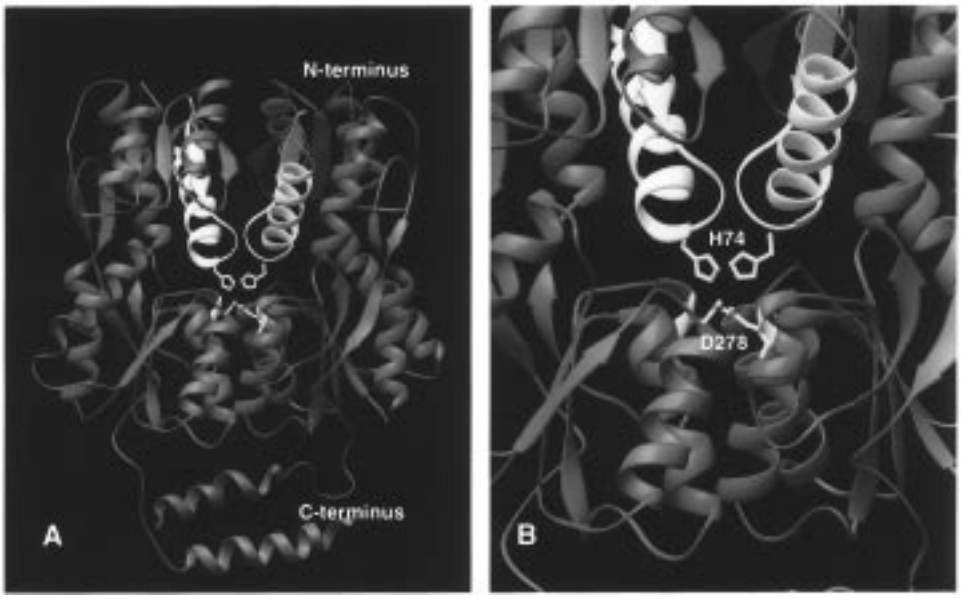

FIGURE 1: *Lac* repressor dimer structures with H74 and D278 backbone residues highlighted. *Lac* repressor inducer-bound core structure was derived from PDB file 1LBH (*14*) and rendered using Ribbons 2.0 (*55*). The N-terminal DNA binding domain is not shown because this region of the protein is not resolved in the crystal structure of the repressor−inducer complex. The N- and C-termini of the protein are labeled, and the backbone residues 62−89 (β-sheet A and helix 5) are shown in white. Intermonomer distance between H74 and D278′ is 2.76 Å, and the B factors for these residues are 30−36 according to the PDB file (*14*), sufficiently low to suggest the possibility of an ion pair interaction. (A) View of entire structure of the dimer unit of the repressor protein. (B) Close up view of the subunit interface near the inducer binding cleft of the structure shown in A.

ture: K84−E100, Q117−R118, and H74−D278 (*14*). The role of these potential ion pairs in stabilizing the protein in the inducer-bound conformation is unknown, and the interactions of these side chains in the operator-bound structure cannot be determined at the current resolution, although the backbone positions corresponding to the H74−D278 pair are sufficiently distant to preclude side chain interaction in the operator-bound structure (*14*).

The H74/D278 interaction is unique in that it involves an amino acid residue in the N-subdomain (H74) with a partner in the C-subdomain (D278) across the monomer−monomer interface. This pair is located in the center of the core domain near the inducer binding site (Figure 1). The intermonomer distance between the H74 and D278 residues is 2.76 Å, a value well within the range for electrostatic interactions. The H74 and D278 residues in the same monomer are 5 Å apart and therefore probably do not form a stable intramonomer interaction. Since the C-subdomain spatial relationships remain constant during the allosteric transition, this intermonomer interaction may be important for stabilizing the N-subdomain in the inducer-bound conformation. Therefore, these residues were specifically substituted to explore their role in *lac* repressor allostery. Substitutions at these positions resulted in distinct changes in both inducer and operator affinities. Depending upon the amino acid substitution, the affinity for these ligands could be enhanced or reduced. Apolar mutations at either residue in this interface resulted in the greatest perturbations of ligand binding, while introduction of aspartate at position 74 or histidine at position 278 to generate a structure with similarly charged residues at both sites did not significantly alter the properties of the mutant protein. Introduction of tryptophan at position 74 resulted in protein that had a severely diminished ability to respond to the presence of inducer. These results suggest that the side chains of amino acid residues H74 and D278 are necessary, but no ionic interaction between these residues is required for wild-type ligand binding behavior.

MATERIALS AND METHODS

Mutagenesis. Mutations in the *lac* repressor gene were generated in the pJC1 plasmid using the method of Kunkel (*33*). All single substitutions were produced by annealing an oligonucleotide containing the mutant sequence to a uracil-containing template with the *lac*I gene. The annealing mixture was heated at 75 °C for two minutes and then allowed to cool to room temperature. The extension and ligation reactions were carried out as described previously (*34*). The double mutant, H74D/D278H, was produced by ligating the *Hind*III−*Apa*I fragment which contains the H74D mutation into the pJCI plasmid with the D278H mutation. The entire *lac*I gene of each mutant produced was sequenced at the core facility at University of Texas Health Science Center (Houston, TX).

Purification. Mutant proteins were expressed in BL-26 cells (Novagen) which were cured of the episome that carries the I^q promoter and the *lac*I gene (Diane Wycuff, personal communication). Protein purification followed the previously described procedure (*35*, *36*). However, the proteins which were not highly expressed (D278H and H74D/D278H) were purified using an additional DEAE chromatography step. The ammonium sulfate pellet was equilibrated in 0.08 M potassium phosphate, pH 7.5, with 5% glycerol. This material was spun at 50000*g* to remove any precipitate and then loaded onto a DEAE column equilibrated in 0.08 M potassium

[1] Abbreviations: DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β,D-thiogalactoside; PurR, purine repressor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

phosphate, pH 7.5. The flow-through from this column was then loaded onto a phosphocellulose column, also equilibrated in the same buffer. Protein concentrations for mutants were determined by absorbance at 280 nm using the extinction coefficient for wild-type *lac* repressor. For the mutant repressor in which an additional aromatic amino acid was added, H74W, a Biorad protein assay using wild-type *lac* repressor as the standard curve was used to determine protein concentration.

Operator Binding. Operator binding constants were determined for the proteins using the nitrocellulose filter binding assay (*37*) modified for use in a 96-well dot blot apparatus (*38*). The assay was carried out at room temperature in FB buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M KCl, 0.1 mM DTT, 0.1 mM EDTA, and 5% DMSO) with 100 μg/mL of bovine serum albumin. The operator used was a 40 base pair double-stranded DNA (sequence: 5′-TGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGG-3′). The concentration of labeled operator in the assay was $\sim 5 \times 10^{-12}$ or 2.5×10^{-13} M for tight binding mutants (*35*). The concentration of protein was varied from 5×10^{-13} to 1.25×10^{-9} M dimer depending upon the affinity of the repressor for operator. The amount of [^{32}P]-operator bound at each protein concentration was quantified as pixels by a Fuji phosphorimager. All data were normalized by dividing the pixels at the different protein concentrations by the pixels at a saturating protein concentration. These data were fit to the following equation:

$$R = \frac{[\mathrm{P}]}{[K_\mathrm{d}] + [\mathrm{P}]} \tag{1}$$

where R is the relative fraction of bound complexes within each solution calculated by R = operator bound/operator bound$_{max}$, [P] is the protein concentration in tetramer, and K_d is the apparent dissociation constant in dimer concentration. The maximum value for R was floated during the fitting.

Nonspecific binding for *lac* repressor proteins was determined by the addition of 1×10^{-3} M IPTG or the use of CS40i, an alternative 40 base pair DNA construct (sequence: 5′-TGTTGTGTGGAGACATGCCTAGACATGCCTTTCACACAGG-3′) that does not contain the *lac* repressor recognition sequence. The labeled DNA concentration in these assays was also $\sim 2.5 \times 10^{-12}$, and the repressor concentration range varied depending on the affinity of the protein for the DNA. The data were collected and analyzed as described for binding to 40 base pair operator DNA.

Inducer Binding. Fluorescence Titration Assay. Inducer binding was assessed by monitoring the change in fluorescence emission intensity (*7*, *39*, *40*). The protein concentration for all mutants was fixed at 5×10^{-7} M monomer, and the IPTG concentration was varied between 1×10^{-8} and 1×10^{-2} M depending upon the affinity of the repressor protein. Proteins were diluted into 0.01 M Tris-HCl, 0.15 M KCl buffer at the specified pH. The tryptophan fluorescence emission was monitored on an SLM 8100 spectrofluorometer using a 340 nm cut-on filter (O-52) from Corning with an excitation wavelength of 285 nm (*7*, *40*). Tryptophan fluorescence in *lac* repressor is dominated by W220, which is located in the inducer binding site. The environment of this fluorophore is altered by inducer binding with a concomitant shift in the wavelength of maximum emission (*39*, *41*−*43*). Fluorescence intensity correction factors were generated using an identical titration with buffer solution instead of IPTG. The binding affinity for the protein was determined by fitting the data points in Igor Pro to the following equation:

$$R = \frac{[\mathrm{IPTG}]^n}{K_\mathrm{d}^n + [\mathrm{IPTG}]^n} \tag{2}$$

where R is the fraction of bound complexes within each solution calculated by R = change in fluorescence signal at [IPTG] divided by the maximum change in fluorescence signal, n is the Hill coefficient, and K_d is the apparent equilibrium dissociation constant for IPTG.

Ammonium Sulfate Precipitation. This assay followed the protocol of Bourgeois (*44*) with the following modifications. The assay buffer contained 0.1 M Tris-HCl, pH 7.4, and 0.15 M KCl, protein stock concentration was 1×10^{-6} M monomer, and operator concentration was 4×10^{-6} M for those samples in which it was included. The protein in varying concentrations was incubated with operator DNA before the addition of 15 mg/mL lysozyme which acted as a coprecipitate, and this mixture was incubated with 5×10^{-7} M [^{14}C]-IPTG for 5 min before precipitation with 70% saturated ammonium sulfate. After these samples were centrifuged, the pellets were resuspended in buffer and the mixtures precipitated with 5% TCA. After centrifugation, the amount of radiolabel in the supernatant was determined by scintillation counting.

Spectroscopic Studies of Protein Unfolding by Urea. Unfolding of repressor proteins by urea was carried out in 0.1 M K_2SO_4, 0.01 M Tris-HCl, pH 7.4, as described previously for dimeric repressor mutants (*45*). Ultrapure urea (Fluka) was prepared fresh daily and filtered before use. The concentration of the urea stock was determined by refractive index. In a denaturation experiment, 15 samples of protein were mixed with the urea stock solution to final urea concentrations between 0 and 6 M and a final protein concentration of 4×10^{-6} M. The final concentration of IPTG was 1×10^{-3} M for those samples which included inducer. Samples were incubated at room temperature for 2 h before the intrinsic tryptophan fluorescence signal was determined using an SLM AB2 spectrofluorometer. The samples were excited at a wavelength of 285 nm, and their fluorescence emission spectra were measured between 300 and 400 nm. The intensity of the intrinsic tryptophan fluorescence signal at 345 nm for unliganded and 325 nm for IPTG-bound repressor protein was used to monitor the protein unfolding.

Circular Dichroism Spectroscopy. Lac repressor mutants were diluted to a concentration of 4×10^{-6} M monomer in 0.12 M potassium phosphate buffer, pH 7.4. Samples were scanned from 250 to 200 nm by an Aviv 62DS spectropolarimeter in a 2 mm path length quartz cuvette. Data were collected for all mutants and compared to the spectrum of the wild-type protein.

Molecular Sieve Column. Lac repressor mutants were applied to a Superose 12 sizing column equilibrated in 0.12 M potassium phosphate buffer, pH 7.4. Elution was monitored by absorbance at 280 nm. All proteins eluted from the column at the same volume as observed for wild-type repressor protein.

Monod−Wyman−Changeux Model Analysis. Inducer binding data for the *lac* repressor proteins were fit to the following equation which expresses the fractional saturation for a tetrameric protein that binds to four inducer molecules and two operator DNA fragments (*7*, *46*):

$$Y = \{K_{RI}[I](1 + K_{RI}[I])^3(1 + 2K_{RO}[O] + K_{RO}^2[O]^2) + LcK_{RI}[I](1 + cK_{RI}[I])^3(1 + 2K_{TO}[O] + K_{TO}^2[O]^2)\}/\{(1 + K_{RI}[I])^4(1 + 2K_{RO}[O] + K_{RO}^2[O]^2) + L(1 + cK_{RI}[I])^4(1 + 2K_{TO}[O] + K_{TO}^2[O]^2)\}$$

In this equation, Y is the fraction of inducer-bound complexes at a specific concentration of inducer, K_{RI} is the equilibrium association constant for the binding of inducer to the R state, and c is the ratio between the equilibrium association constants for the T and R states for inducer (K_{TI}/K_{RI}) and reflects the difference in inducer affinity for these two states. K_{RO} is the equilibrium association constant for operator DNA binding to the R state, and K_{TO} is the equilibrium association constant for the T state. The equilibrium constant, L, represents the relative concentration of the T and R states when no ligand is present (L = [T]/[R]). Inducer binding data for repressor proteins in the presence and absence of operator DNA were fit simultaneously in Non Lin (*47*) varying only the value of L.

RESULTS

Generation of Mutants and Protein Purification. Five substitutions at residue H74 (A, L, D, F, W), seven substitutions at D278 (A, L, H, N, E, K, W), and a double substitution (H74D/D278H) were made to explore the importance of this amino acid pair in *lac* repressor allostery. The entire *lac*I gene of each mutant was fully sequenced to determine that no other alterations were present. All mutant repressors were expressed in *Escherichia coli* with the genomic *lac*I gene deleted and were purified by phosphocellulose column chromatography. The D278W mutant was not expressed at levels sufficient for purification. The D278H and the H74D/D278H mutants also were poorly expressed but could be purified with an additional DEAE chromatography step. Circular dichroism spectra for all repressors demonstrated the same secondary structure content as wild-type repressor within experimental error (data not shown). On the basis of elution behavior from the phosphocellulose column and from a molecular sieve column, all mutant proteins were deduced to be tetrameric (data not shown).

Operator Binding. All proteins with substitutions for H74 or D278 bound to operator sequences specifically as shown in Figure 2, and the derived equilibrium dissociation constants are listed in Table 1. Mutation of H74 to apolar residues altered operator binding with the effect related to the size of the substitution. Substitution with smaller apolar amino acids (A or L) decreased operator binding, while larger amino acids (W or F) increased operator affinity. The H74D mutant repressor displayed essentially wild-type affinity for operator binding. All of the D278 substitutions resulted in increased affinity for operator. The double mutant protein, H74D/D278H, had a slightly reduced affinity for operator, similar to the H74D mutant repressor.

In the presence of inducer, all mutant proteins except for the H74W protein exhibited significantly decreased affinity for operator sequences (examples in Figure 3; Table 1). The H74W mutant repressor bound operator with high affinity even when complexed with inducer. Its affinity for operator in the presence of inducer was decreased only ∼10-fold to 6.3×10^{-11} M. Increased incubation times and higher concentrations of inducer did not alter these results. This property is not a result of the H74W protein binding more tightly to DNA in general, as its binding affinity for a 40 base pair nonoperator DNA sequence was comparable to wild-type repressor bound either to the nonoperator DNA or to operator DNA in the presence of inducer (Figure 4).

Inducer Binding. Inducer binding for this series of *lac* repressor mutants was measured by the change in tryptophan fluorescence upon IPTG binding. At neutral pH, the *lac* repressor equilibrium constant for wild-type protein binding to IPTG is 2.5×10^{-6} M. However, in the presence of operator the affinity for inducer is decreased ∼7−10-fold (*5*−*7*, *46*). For wild-type, the same ∼7−10-fold decrease is mimicked at pH 9.2 (*46*, *48*, *49*). The inducer binding affinity of H74 and D278 mutant repressors was assessed at neutral and high pH and in the presence of operator to determine if these binding processes were affected. The curves for repressor mutants are shown in Figure 5, and the K_d values derived from these curves for all mutant proteins are provided in Table 2. At neutral pH, substitutions at H74 resulted in slightly tighter or weaker inducer binding depending upon the size of the residue. The smallest amino acid substitution, A, resulted in a slightly higher affinity than wild-type protein, while L at position 74 diminished binding. The H74D repressor, which has two negatively charged residues in close proximity at the subunit interface, displayed essentially wild-type inducer binding. In contrast, a large, apolar amino acid substitution at this position, F, resulted in an ∼8-fold decrease in inducer affinity.

At elevated pH, the smaller amino acid substitutions did not show the characteristic shift in inducer affinity, while the H74F repressor displayed a ∼7-fold decrease in affinity compared to its affinity in neutral pH, behavior which is more similar to that of the wild-type protein (Figure 6). In the presence of operator, inducer affinity was reduced for all of the H74 mutant repressors relative to their affinity at neutral pH. Both H74A and H74L repressors had slightly decreased affinity for IPTG in the presence of operator, while the affinity of the H74D protein for IPTG was decreased by ∼4-fold under these conditions. The H74F repressor had a greater relative shift (∼13-fold) for inducer binding with operator present compared to wild-type protein (∼7-fold). These results indicate that different amino acid substitutions at this position alter inducer affinity and the effect that operator and high pH have on inducer affinity. Because elevated pH significantly alters the inducer affinity of the H74F repressor, the histidine side chain at residue 74 is clearly not the sole determinant of the elevated pH effect.

All residues substituted for D278 resulted in decreased affinity for inducer at neutral pH. The D278A and D278N mutant repressors displayed a ∼5-fold decrease, while D278H, D278E, and D278K mutant repressors showed only a ∼2-fold decrease. All mutant repressors displayed a further decrease in affinity at elevated pH and a relative ∼10-fold decrease in affinity in the presence of operator compared to their affinity at neutral pH. Therefore, the pH-dependent properties of the protein were less sensitive to the nature of

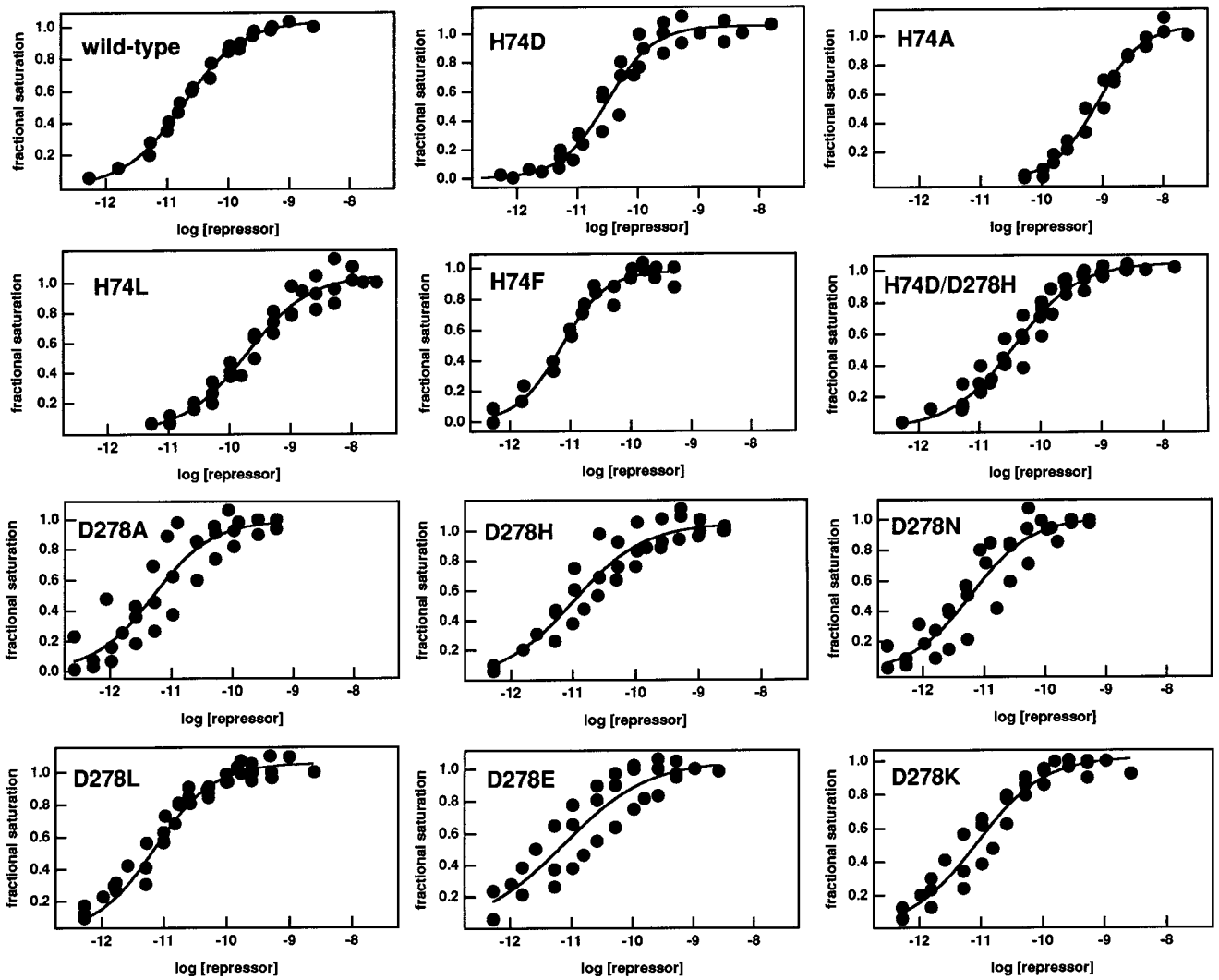

FIGURE 2: Operator binding curves for mutant repressor proteins. The concentration of labeled DNA was 2×10^{-12} or 5×10^{-13} M depending upon the affinity of the repressor protein, and protein concentration is reported as dimer. Assays for repressor binding to 40 base pair operator sequence were performed a minimum of three times. The curves were generated by simultaneously fitting all the data to eq 1 in Materials and Methods. Relative fractional saturation was determined as the ratio of labeled DNA retained on nitrocellulose for a specific concentration of repressor to labeled DNA retained at saturating concentrations of repressor. H74W data are shown in Figure 4.

the substitution at residue 278 in comparison with the H74 substitutions. Since all side chain alterations resulted in a decrease in affinity at elevated pH, the presence of D278 in the interface is not essential for this allosteric effect. Interestingly, the double mutant repressor, H74D/D278H, did not re-establish wild-type inducer binding behavior for the repressor protein. While H74D/D278H protein had a slightly reduced affinity for inducer at neutral pH, elevated pH resulted in inducer affinity comparable to wild-type protein at neutral pH. The presence of operator decreased inducer affinity only ~2-fold, a degree comparable to that observed for the smaller substitutions at H74.

Because the additional tryptophan altered its emission properties, the fluorescence assay could not be used to determine inducer affinity for H74W repressor; therefore, an ammonium sulfate precipitation assay was used to determine the relative affinity of the protein for inducer. Since no change in inducer affinity at elevated pH was detected for wild-type protein in this assay, presumably due to the high ammonium sulfate concentrations required, this method could not be used to determine the effect of high pH on inducer affinity. The H74W mutant displayed the same affinity as the H74F mutant for inducer at neutral pH and in the presence of operator (Figure 7). The H74A mutant protein, which only has a 2-fold reduced affinity for inducer in the presence of operator, did not show a change in affinity in this assay (data not shown). Since the H74F and H74W repressors behave similarly in the ammonium sulfate precipitation assay, we conclude that their inducer binding affinities are similar in both the absence and presence of operator DNA.

The affinity of the D278L repressor for inducer could not be determined because this protein precipitated noticeably upon binding IPTG. Although other substitutions at this site resulted in a tendency to precipitate at high protein concentrations in the presence of inducer, this phenomenon was most severe for the D278L mutant protein. The amount of precipitation was dependent on both protein and IPTG concentrations. Fluorescence titration assays indicated that the D278L repressor had a ~100-fold decrease in IPTG binding affinity compared to wild-type protein (data not shown), although the precision of these results is questionable due to potential precipitation even at the lower concentrations employed in this assay.

Table 1: Operator Binding Properties of Mutant Repressors

repressor	K_d[a] operator binding $\times 10^{11}$ M	K_d[a] operator binding + IPTG[b] $\times 10^{11}$ M
wild-type	1.8 ± 0.10	4800 ± 2200
H74A	50 ± 8.5	3800 ± 530
H74L	19 ± 2.9	3000 ± 500
H74D	3.1 ± 0.40	4200 ± 470
H74F	0.72 ± 0.07	2000 ± 430
H74W	0.50 ± 0.03	6.4 ± 1.1
H74D/D278H	3.8 ± 0.44	1400 ± 320
D278A	0.51 ± 0.14	9900 ± 2800
D278L	0.72 ± 0.07	nd[c]
D278N	0.60 ± 0.15	2800 ± 850
D278H	1.0 ± 0.22	1500 ± 260
D278E	0.73 ± 0.26	26000 ± 6500
D278K	0.86 ± 0.15	20000 ± 5500

[a] The equilibrium dissociation constants of the *lac* repressor for 40 base pair operator DNA were measured using nitrocellulose filter binding assays as described in Materials and Methods. Each value was determined by fitting at least three curves simultaneously to eq 1 as described in Materials and Methods. Error values were generated by Igor Pro and represent the standard deviation of the fitted curve. The values are determined using the concentration of repressor dimer to represent the affinity of a single binding site. [b] Equilibrium dissociation constants were measured in the presence of 1×10^{-3} M IPTG. Operator concentration for these assays was $\sim 2 \times 10^{-12}$ M. [c] nd = not determined.

One rationale for the precipitation of D278L upon binding IPTG is that occupation of the inducer binding site destabilizes the structure. Urea denaturation was monitored using tryptophan fluorescence to determine if the precipitation was a result of the mutant protein being less stable when bound to IPTG. The emission wavelength maxima of both wild-type and D278L repressor proteins are shifted to shorter wavelength upon binding IPTG, and this shift in the wavelength of maximum fluorescence emission was used to establish that the D278L protein remained bound to IPTG at different concentrations of urea (Figure 8). The fluorescence intensity for D278L as a function of urea was compared to wild type both in the presence and absence of IPTG (Figure 9). The D278L repressor displayed slightly diminished stability compared to wild-type protein in this assay; however, both of the repressors appeared more stable when bound to IPTG than the corresponding, unliganded repressor. This shift in stability was slightly greater for the D278L protein compared to wild-type protein. Therefore, IPTG binding does not markedly decrease the stability of the D278L mutant repressor, and the precise cause of precipitation remains unclear.

Monod−Wyman−Changeux Model of Allostery. Traditionally, *lac* repressor allostery has been described using the Monod−Wyman−Changeux (MWC) model (*50*) for concerted allosteric transitions between distinct ligand binding conformations of the protein (*7*, *46*). This model assumes that the protein possesses different ligand binding conformations (R and T forms) that have either a higher or lower affinity for the ligand, and that the equilibrium between these conformations alters the apparent affinity of the protein for the ligand. Therefore, alterations in ligand binding are expressed as changes in the equilibrium between the different conformations, and this value is called L, the allosteric constant of the protein (= [T]/[R]). This analysis presumes that amino acid substitutions alter the equilibrium between R and T forms of the protein (i.e., L) but do not alter the thermodynamics of inducer or operator binding directly (i.e., the intrinsic binding constants K_{TO}, K_{RO}, K_{TI}, and K_{RI} are unchanged), an assumption that may not apply for every mutant examined. Nonetheless, this analysis is useful in conceptualizing the effects of mutations generated in this study. Wild-type repressor has an allosteric constant near unity (*7*, *46*). This value indicates that this protein exists in an equilibrium that favors equally the two ligand-bound states.

To fit binding data using the MWC model, one must know the intrinsic binding constants. Some *lac* repressor mutants have tighter binding constants for either inducer or operator than wild type (*28*, *51*). Whether the behavior of these mutant repressors reflects the intrinsic binding constants for different states of the protein or results from an alteration of binding site thermodynamics has not been established. The intrinsic affinity for IPTG can be determined because monomeric mutant repressors retain this activity with kinetic and thermodynamic parameters similar to those of the wild-type tetrameric repressor (*52*). However, since the dimer structure is necessary for operator binding, and the stability of the dimer influences the apparent equilibrium constant for operator binding, the intrinsic operator binding constant is more difficult to determine (*45*).

By using the Monod−Wyman−Changeux model, one could fit inducer binding in the absence and presence of operator for the H74 and D278 mutant repressors (Figures 5 and 6) to one set of ligand binding parameters varying only the allosteric constant. The binding isotherms in Figures 5 and 6 were fit simultaneously to determine the allosteric constant (L) for each mutant using the intrinsic binding constants for wild-type repressor (*7*, *46*) rather than the extreme values observed for mutant proteins, which may result from altered contacts between ligand and protein. The allosteric constants generated from these fits are shown in Table 3. The error ranges for the derived L values indicate the degree to which this model is appropriate for each mutant, and we conclude from the nature of the fits and error ranges that application of the MWC model to H74L and H74D/D278H is not appropriate. Despite the underlying assumptions and inherent limitations in applying this model to mutant proteins, the derived values indicate that mutations at H74 can result in stabilizing the equilibrium toward either inducer-bound or operator-bound conformation depending upon the size of the substitution at this residue. The equilibria of the D278 mutant repressors were all shifted toward the operator-bound state to varying degrees.

DISCUSSION

A comparison of X-ray crystallographic structures of *lac* repressor in different ligand-bound forms demonstrates a reorientation of the N-subdomain in the monomer−monomer interface between these states of the protein (*14*). H74 is located at the beginning of helix 5 near the inducer binding cleft (Figure 1). The secondary structural element before helix 5 is β-strand A, which is the first β-strand in the core domain of the protein. This β-sheet attaches the DNA binding region to the core via the hinge helix. Clustered at the beginning of helix 5 are several amino acids important for forming hydrophobic contacts with IPTG in the inducer-bound crystal

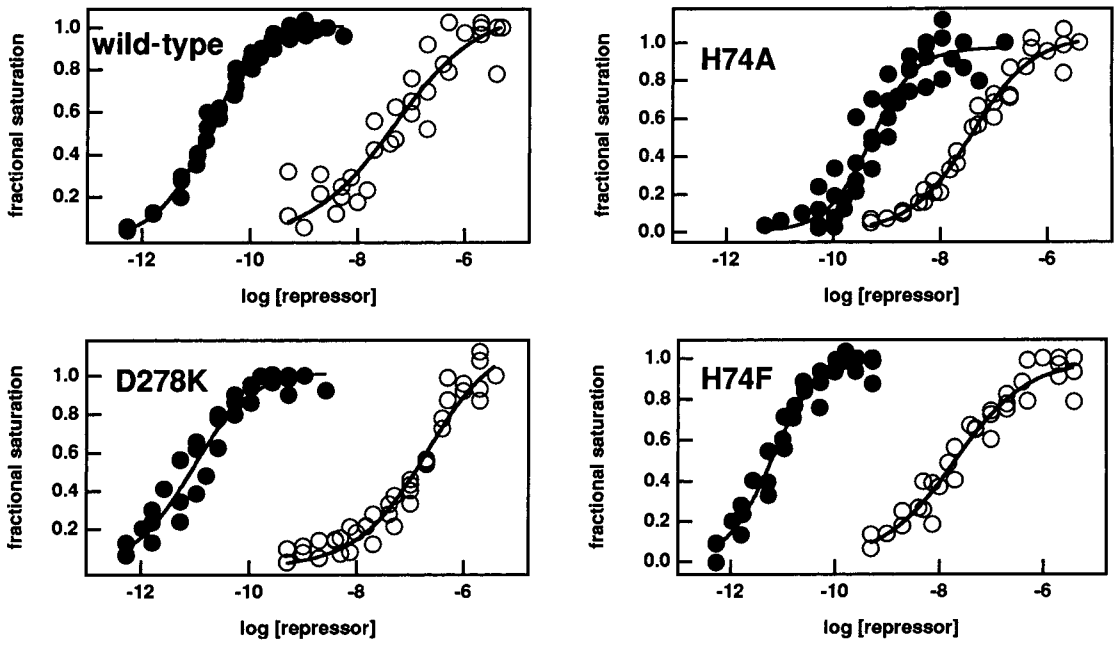

FIGURE 3: Comparison of operator binding curves for repressor proteins in the absence and presence of IPTG. The binding of repressor protein to a 40 base pair operator sequence in the presence of 1×10^{-3} M IPTG (○) was determined in the same manner as the unliganded protein (●) in Figure 2 except for changes in the range of protein concentrations assayed. A selection of mutant proteins that represent the different patterns observed for operator binding is shown. Assays for binding in the presence of IPTG were performed a minimum of three times, and the curves were generated by simultaneously fitting all the data to eq 1 in Materials and Methods.

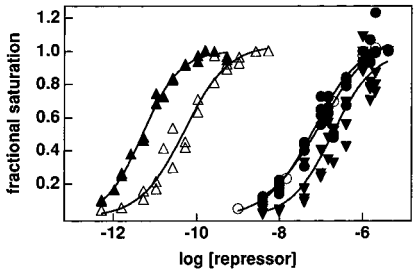

FIGURE 4: H74W repressor protein binding to different operator constructs. The affinity of H74W repressor protein for 40 base pair operator sequence in the absence (▲) and presence (△) of 1×10^{-3} M IPTG was determined by nitrocellulose binding. The binding of the repressor to a nonspecific 40 base pair sequence (CS40i) (▼) was also determined for H74W protein. Binding curves for wild-type repressor to CS40i (●) and to 40 base pair operator in the presence of IPTG (○) are also included. The samples that contained IPTG were incubated for greater than 2 h with 1×10^{-3} M IPTG.

structures (*13*, *14*). Therefore, H74 is located at the end of the strand which connects the core with the DNA binding region and is also located near a cluster of amino acids which are important for inducer contacts. This location potentially allows H74 to play a role in stabilizing the proper contacts for inducer binding. The location of D278 in the C-subdomain, which does not change its orientation upon inducer binding, would anchor the H74 residue in the proper conformation for high-affinity inducer binding. Previous genetic screens of *lac* repressor have shown that mutations in this region result in altered ligand binding behavior, even though these areas do not correspond with the proposed ligand binding sites (*11*, *12*, *29*, *53*). Presumably, mutation of these residues alters ligand binding due to effects on the allosteric transition of the protein, either by perturbing structural transitions between different conformations or by affecting the forces which stabilize or destabilize the ligand-bound conformations.

On the basis of its location in the monomer−monomer interface spanning the N- and C-subdomains across the subunit interface, the H74−D278 interaction is in a unique position to influence the conformational stability of the inducer-bound form. Therefore, the importance of this interaction for *lac* repressor structure and function was explored by mutational analysis. Dramatically different effects on both ligand binding and allosteric properties of the mutant repressors were observed on the basis of the nature of the amino acid substituted for residue H74. The results from the multiple mutations made at this residue indicate that the size of the substitution is important. The larger amino acids, F and W, both caused a decrease in inducer affinity and higher affinity for operator. The smaller amino acids, A, L, and D, displayed the opposite behavior. The latter three substitutions all resulted in decreased operator binding and approximately wild-type affinity for inducer. The hydrophobicity of the substitution did not seem to be as crucial, because L and F substitutions exhibited different binding characteristics. However, as observed previously (*36*), introduction of the bulky side chain of tryptophan at position 74 resulted in a drastic reduction in the allosteric response to inducer. This alteration resulted in only a ~10-fold decrease in operator DNA binding affinity in the presence of inducer, although the nonspecific affinity for DNA was unaltered. The D mutation, for which the similar charge should have prevented interaction with D278 across the monomer−monomer interface, demonstrated the least change in ligand binding. One reason for this observation could be that the size of D is the most similar to H. From these data, we conclude that the size of the amino acid at H74 is the most important factor for allosteric properties rather than the formation of a salt bridge.

Substitutions at D278 did not have effects on allostery as severe as mutations at residue H74; all D278 mutant

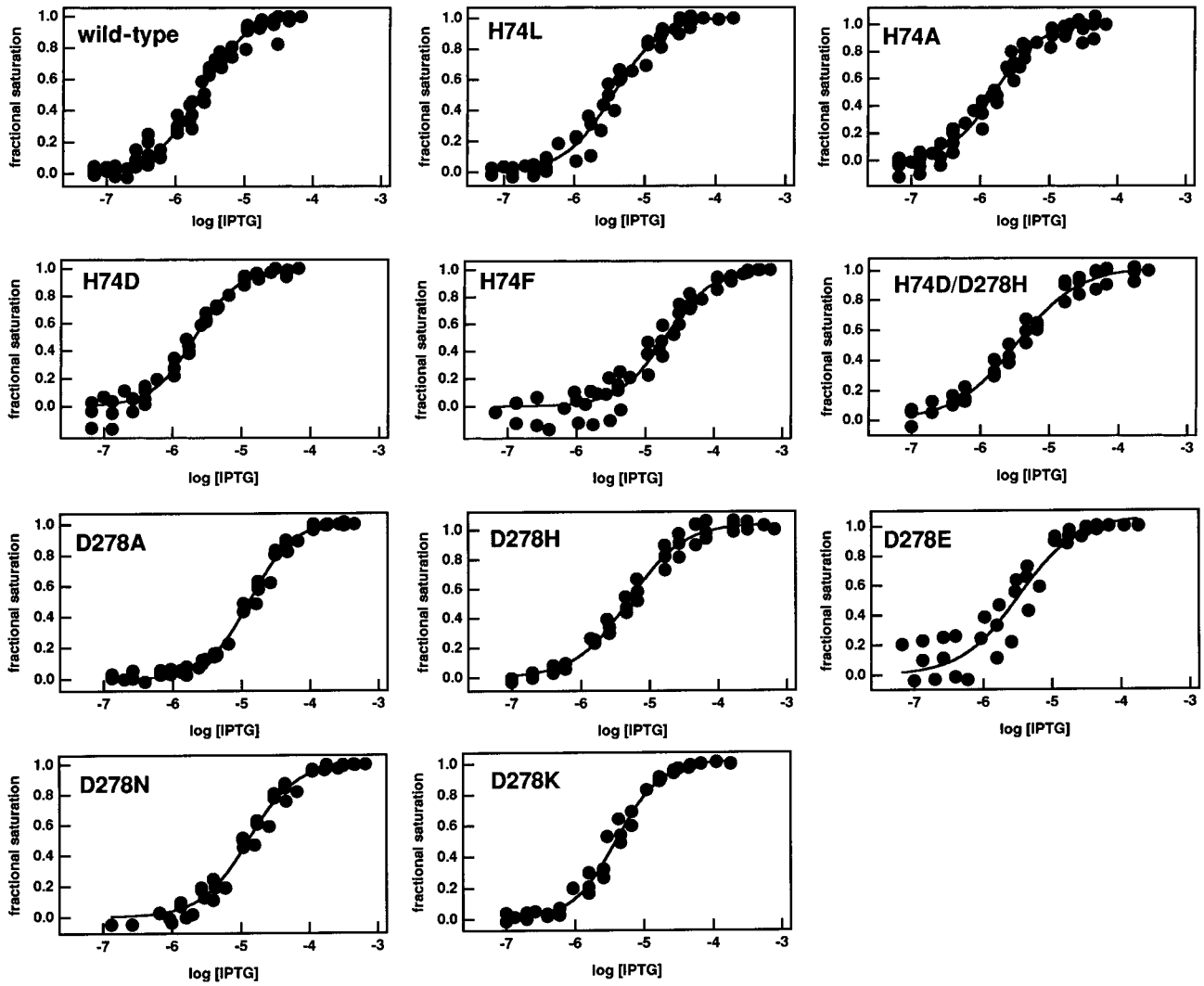

FIGURE 5: IPTG titration curves for *lac* repressor proteins. Fluorescence titrations were performed on an SLM 8100 spectrofluorometer as described in Materials and Methods. The concentration of protein was 5×10^{-7} M monomer in 0.01 M Tris-HCl (pH 7.4), 0.15 M KCl buffer. The curves were generated by simultaneously fitting at least three replicates to eq 2.

Table 2: Inducer Binding Properties of Mutant Repressors

	K_d[a] IPTG binding		
repressor	pH 7.4 ($\times 10^6$ M)	pH 9.2 ($\times 10^6$ M)	+ operator DNA[b] ($\times 10^6$ M)
wild-type	2.5 ± 0.1	16 ± 0.1	17 ± 0.1
H74A	2.0 ± 0.1	2.0 ± 0.01	3.5 ± 0.02
H74L	3.6 ± 0.3	4.0 ± 0.02	8.0 ± 0.05
H74D	2.1 ± 0.05	2.8 ± 0.02	8.9 ± 0.07
H74F	20 ± 1.6	140 ± 1.1	270 ± 3.1
H74W	nd[c]	nd	nd
H74D/D278H	3.5 ± 0.03	2.1 ± 0.02	7.3 ± 0.08
D278A	14 ± 0.4	71 ± 0.33	210 ± 1.9
D278L	nd	nd	nd
D278N	13 ± 0.7	72 ± 0.64	130 ± 1.4
D278H	5 ± 0.3	10 ± 0.08	60 ± 0.4
D278E	4.2 ± 1.7	18 ± 0.3	38 ± 0.7
D278K	4.6 ± 0.2	18 ± 0.2	36 ± 0.6

[a] Equilibrium dissociation constants for IPTG binding were determined using fluorescence titration experiments. Protein concentration was 5×10^{-7} M monomer. Values were determined by simultaneously fitting at least three independent data sets to eq 2 in Materials and Methods. [b] Samples with operator DNA contained 1×10^{-6} M 40 base pair operator fragment. [c] nd = not determined.

repressors displayed a similar decrease in operator binding in the presence of IPTG. The IPTG binding affinity of all substitutions at D278 was reduced by high pH and in the presence of operator compared to their affinity in neutral pH. Therefore, the potential salt bridge is not necessary for sensitivity of inducer binding to operator or high pH. However, mutations in this region nonetheless altered the ligand binding properties of the repressor, with increased operator binding affinity and reduced inducer binding affinity. The alteration in ligand binding affinity can be explained on the basis of the allosteric equilibrium of the protein being shifted toward the operator-bound form. While mutations at the D278 residue did not abolish allosteric behavior, D at this position appears to be important for maintaining wild-type ligand binding, in particular operator affinity. Increased affinity for operator sites, as observed for these mutant repressors, could affect the ability of the bacterium to respond effectively to lactose availability.

Substitution of other subunit interface residues in the regions involved in allosteric transitions has been shown to result in altered inducer and operator binding affinities. Mutation of A110, located in helix 6, yielded effects on both inducer and operator ligand binding properties that were dependent upon the nature of the substitution (*51*). These results were also interpreted in the context of different

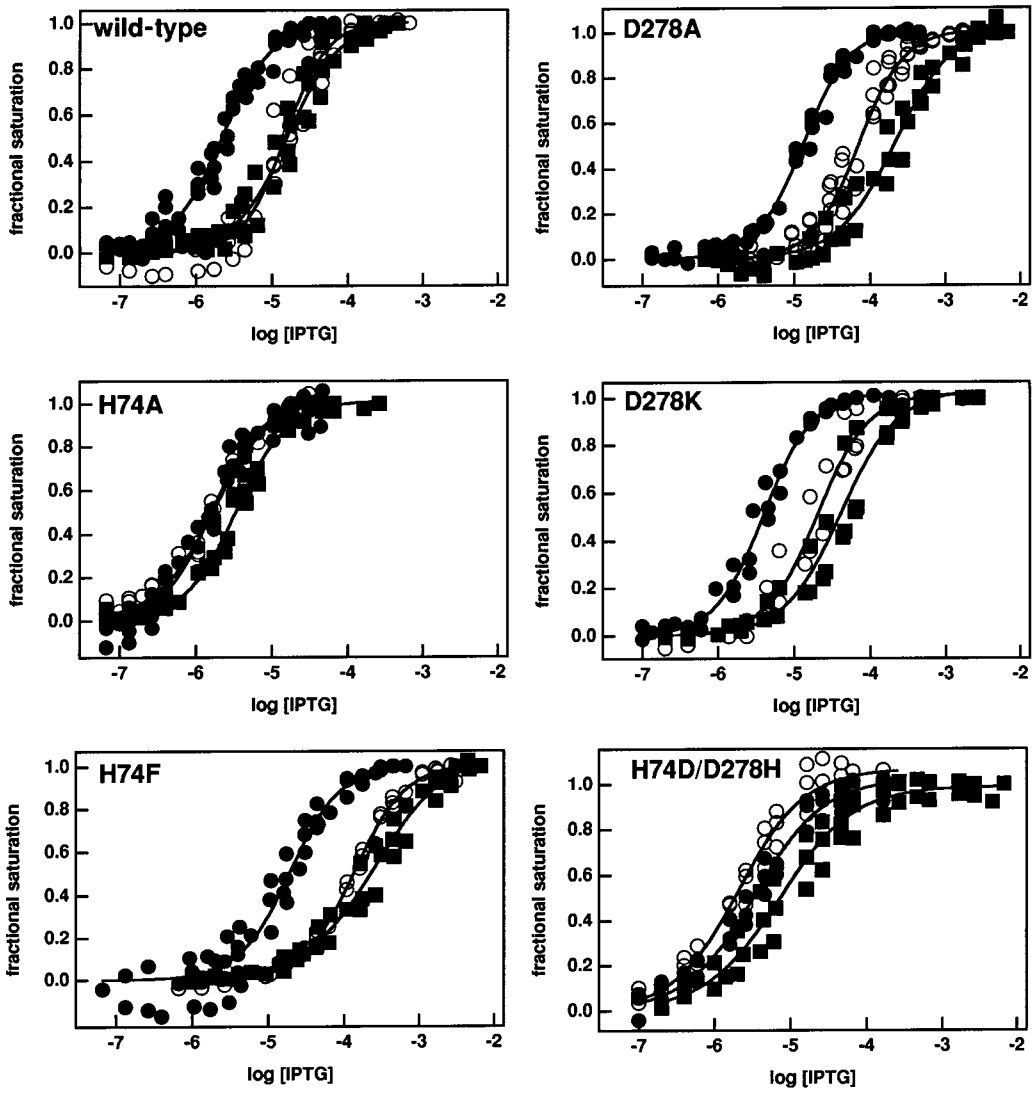

FIGURE 6: Comparison of IPTG titration curves for repressor proteins under different conditions. A selection of mutant proteins that represent the IPTG binding behavior of repressor proteins under different conditions is shown: pH 7.4 (●), pH 9.2 (○), or in the presence of 40 base pair operator DNA (■). Samples with DNA contained 1×10^{-6} M 40 base pair operator. Except for these changes, the assays were performed and analyzed as described in Figure 5.

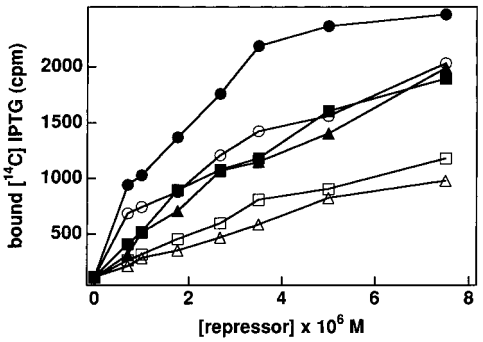

FIGURE 7: IPTG binding measured by ammonium sulfate assay. Binding was measured as described in Bourgeois (*44*) with modifications listed in Materials and Methods. Curves are drawn through the data for comparison purposes only. The different proteins are as follows: wild type (●), H74W (▲), and H74F (■). Open symbols represent samples which include 1×10^{-6} M 40 base pair operator DNA.

substitutions stabilizing the repressor in either the inducer- or operator-bound conformation. Another residue that has been substituted and the protein characterized in vitro with alterations in both operator and inducer binding is D88 (*28*). Substitution of K for D88, located in helix 5, resulted in a decrease in inducer binding and an increase in operator binding affinity. Modification of subunit interface residues not located in regions where structural changes are observed upon ligand binding also has resulted in binding behavior that can be explained by alterations in the allosteric equilibrium of the modified repressor. The modification of C281 with methyl methanethiosulfonate (MMTS) generated a decrease in operator binding affinity (*40*). The complete ligand binding behavior of this protein could be fit to an allosteric model where the protein was stabilized in the inducer-bound state. Thus, specific residues in the subunit interface exert significant influence over the allosteric equilibrium of *lac* repressor, and substitutions or modifications of these amino acid residues can alter this equilibrium.

The inability to express the D278W mutant repressor, while the H74W protein could be purified, is an indication of the differences in steric limitations between the N- and C-subdomain monomer−monomer interfaces. From an analysis of the crystal structure, the N-subdomain interface, which must have the flexibility to rearrange upon ligand binding,

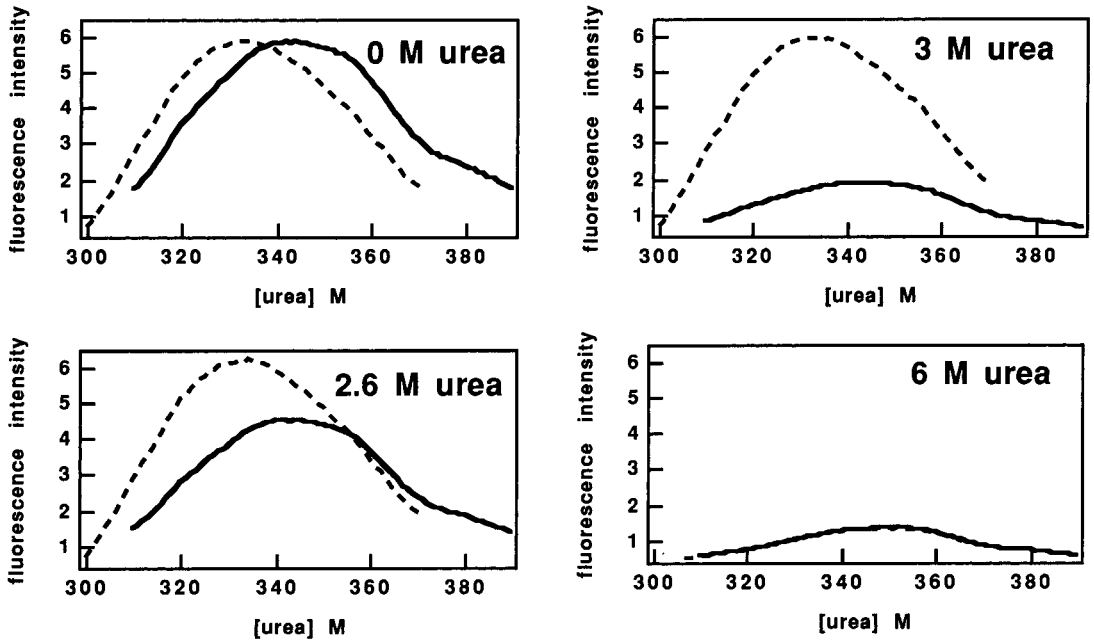

FIGURE 8: Fluorescence emission scans of D278L protein in the absence and presence of inducer at various concentrations of urea. Fluorescence emission scans were collected on an SLM AB2 spectrofluorometer using an excitation wavelength of 285 nm. The repressor samples were diluted to 4×10^{-6} M monomer concentration in 0.01 M Tris-HCl, pH 7.4, 0.1 M K_2SO_4 buffer at the indicated concentrations of urea. Spectra represented by dashed lines indicate that 1×10^{-3} M IPTG was present. The difference in the wavelength maximum between the unliganded versus the inducer-bound samples demonstrates that the protein is bound to IPTG even in the presence of urea.

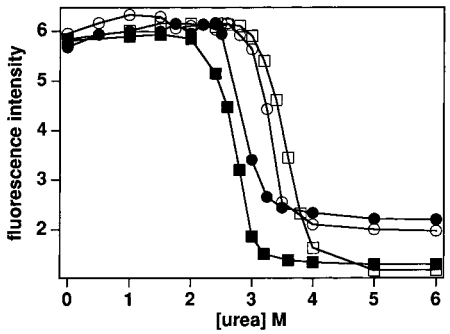

FIGURE 9: Urea denaturation of D278L repressor protein. Relative stability of D278L in the absence (■) and presence of IPTG (□) was determined by monitoring the unfolding of the protein as a function of urea concentration by fluorescence signal. The denaturation data for wild-type protein in the absence (●) and the presence of IPTG (○) are included for comparison. Curves are drawn through the data for comparison purposes only. Protein concentration was 4×10^{-6} M monomer, and samples with inducer present were preincubated with 1×10^{-3} M IPTG. Samples were excited at 285 nm, and the fluorescence emission intensity at 345 nm for unliganded or 325 nm for IPTG-bound protein was recorded using a SLM AB2 spectrofluorometer.

Table 3: Allosteric Constants for Mutant Repressors[a]

repressor	allosteric constant (L)[b]	error range
wild-type	0.73	(0.56−0.96)
H74A	0.0006	($5-8 \times 10^{-4}$)
H74D	0.013	($9 \times 10^{-3}-2 \times 10^{-2}$)
H74L	0.009	($6 \times 10^{-3}-2 \times 10^{-2}$)
H74F	40000	($3-6 \times 10^{4}$)
H74D/D278H	0.0063	($4 \times 10^{-3}-9 \times 10^{-2}$)
D278A	9700	($8 \times 10^{3}-1 \times 10^{4}$)
D278H	79	(58−100)
D278N	9500	($7 \times 10^{3}-1 \times 10^{4}$)
D278E	13	(8.6−19)
D278K	25	(17−37)

[a] Inducer binding curves (Figures 5 and 6) in the absence and presence of operator were fit to the Monod−Wyman−Changeux model using NonLin (*47*). The two different data sets for each repressor mutant were fit using the same intrinsic binding constants and varying only the allosteric constant (L). The intrinsic binding constants for these fits were $K_{RI} = 7.1 \times 10^5$ M^{-1}, $K_{RO} = 6.3 \times 10^6$ M^{-1}, $K_{TO} = 1.7 \times 10^9$ M^{-1}, and $c = 0.011$ (*46*). [b] $L = [T]/[R]$.

is less constrained than the C-subdomain of the monomer−monomer interface, which remains in a fixed orientation upon ligand binding (*14*). Differences in the steric constraints present in the N- and C-subdomain monomer−monomer interface are demonstrated by a comparison of the residues surrounding the H74 and D278 residues. The only inter-monomer contact within 6 Å for the H74 residue is D278. However, within 6 Å the D278 residue is surrounded by residues S221, A222, Q248, L251, and Y282, as well as H74 of the opposite monomer. The lack of steric constraints surrounding H74 may be responsible for the dramatic alteration in the effect of inducer binding on the operator affinity for H74W repressor. The substitution of a bulky side chain at the N-subdomain subunit interface may introduce a steric constraint that precludes the structural shifts required to rearrange the N-subdomains and the DNA binding site in this mutant protein. The inability to generate D278W and precipitation of the D278L repressor in the inducer-bound form may reflect the additional constraints experienced by the C-subdomain interface. The larger, charged amino acids, such as K, E, and H, may be tolerated at this position because they are more polar and can interact with the solvent when excluded from the subunit interface.

If the loss of a putative H74−D278 salt bridge were responsible for the decrease in inducer affinity at high pH, mutation of either of these residues would result in no change in inducer affinity at high pH compared to the affinity of the mutant repressor at neutral pH. However, one of the H74 and all of the D278 mutant proteins showed changes in inducer affinity at high pH compared to their neutral pH

affinity. The magnitude of the decrease in affinity at high pH compared to neutral pH varied for the different mutant repressors. The D278 mutant repressors all displayed an almost wild-type decrease in inducer affinity at elevated pH. No H74 substitutions, except for the H74F repressor, displayed a significant decrease in inducer binding at high pH. The H74F mutant protein displayed a ~7-fold decrease in inducer affinity at high pH. High pH actually restored wild-type inducer affinity for the double mutant repressor protein, H74D/D278H. Elevated pH reduces the inducer binding affinity of the wild-type repressor, presumably via a quaternary conformational change, with results similar to those caused by binding operator (*46*). However, the additional decrease in inducer affinity when the protein is bound to operator at high pH indicates that these two allosteric processes do not share identical mechanisms (*46*). Mutation of subunit interface residues in either the N- or C-subdomains reduces the effect of elevated pH on inducer binding. In the N-subdomain, mutations of K84 and D88 resulted in only 2- and 4-fold decreases in inducer binding at high pH (*27*, *28*). In the C-subdomain, the size of the substitution at C281 correlated with alterations in inducer binding affinity at elevated pH (*26*). These results indicate that multiple factors, such as charge and size of the residues in the subunit interface, affect the decrease in inducer binding observed at high pH.

High pH effects on inducer binding correlate with the influence of operator on inducer binding. In general, those mutants with a decrease in inducer affinity at high pH showed a larger decrease in inducer binding in the presence of operator. However, even the H74 mutant repressors that showed no decrease in affinity at high pH still have a slight (~2-fold) decrease in the presence of operator. These results indicate that there are probably certain elements, such as subunit interface spacing, that reduce inducer binding both at high pH and in the presence of operator. There seem to be other factors, such as the presence of charged amino acids in the subunit interface, that may only be important for the allosteric effect of protons on inducer binding. The relative importance of interplay between these different factors may account for the restoration of the high pH effect in the H74F repressor, perhaps achieved by altering the spacing of the interface to compensate for the loss of the imidazole side chain. Detailed structural analyses of the mutant proteins will be necessary to address these issues further.

The potential salt bridge between the two interface residues, H74 and D278, does not appear to contribute to the formation or stabilization of either ligand-bound conformation, since the *identity* of the residue at either site appears responsible for the observed influences on ligand binding properties. Ion pairs found in the hydrophobic core of proteins usually do not contribute to the energy of folding or stability because the entropic cost of satisfying their hydrogen bonding needs is high (*54*). These interactions usually form when charged residues are necessary in a hydrophobic region of the protein for function. The failure of the double mutant protein, H74D/D278H, to restore wild-type function may result because the potential salt bridge plays no unique function in the interface. Nonetheless, the identity of the residues at H74 and D278 affects the conformational equilibrium of the repressor protein, and charged residues in this interface may be necessary to maintain the flexibility needed for the allosteric transition to occur.

These studies link amino acids identified in regions that differ between the two ligand-bound conformations of *lac* repressor with a functional role in allostery. A similar pair of charged residues interacting across the subunit interface from the N- and C-terminus was also found in the PurR structure (*31*). The presence of these similar pairs of interacting residues in two members of this family of proteins indicates that they may contribute to the allosteric properties required for the function of these regulatory proteins. Mutations of amino acids located in subunit interfaces involved in conformational changes induced by ligand binding advance our currently limited understanding regarding how specific structural elements contribute to the functional aspects of allostery.

NOTE ADDED IN PROOF

After submission of this article, Lu et al. (Lu, F., Brennan, R. G., and Zalkin, H. (1998) *Biochemistry 37*, 15680−15690) reported that the homologous interdomain salt bridge observed between E70 and R278′ in purine repressor is not crucial for stabilizing the closed conformation of PurR. Effects on allosteric equilibrium were also observed with substitution of E70 in PurR as found for its homologue, H74, in the present study for LacI.

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