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Allostery and Assembly of $\textit{Lac}$ Repressor Protein

by

Jennifer K. Barry

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE

Kathleen Matthews
Kathleen S. Matthews, Stewart Memorial Professor of Biochemistry and Cell Biology
Dean of Wiess School of Natural Sciences

John S. Olson, Dorothy and Ralph Looney Professor of Biochemistry and Cell Biology

George Bennett
George N. Bennett, Professor of Biochemistry and Cell Biology

Edward P. Nikonowicz, Assistant Professor of Biochemistry and Cell Biology

Scott F. Singleton, Assistant Professor of Chemistry

August, 1998
Abstract

Allostery and Assembly of *Lac* Repressor Protein

by

Jennifer K. Barry

Allostery in *lac* repressor ligand binding is based upon the ability of the protein to assume alternate ligand-bound conformations. The X-ray crystal structures of repressor bound to different ligands indicate the end points of conformational transitions. To detect changes in the environment within the repressor upon ligand binding, single tryptophan substitutions were generated in the following locations: the N-terminus (Tyr$^7$); the junction between the N-terminus and the N-subdomain (Leu$^{62}$); the N-subdomain of the monomer-monomer interface (Glu$^{100}$ and Gln$^{117}$); the central region of the core (His$^{74}$, Tyr$^{273}$, and Phe$^{293}$); the C-subdomain (Phe$^{226}$); and the C-terminus (Lys$^{325}$). Changes in fluorescence properties upon inducer binding are only detected for tryptophans substituted at His$^{74}$, Tyr$^{273}$, and Phe$^{293}$. Fluorescence properties are not altered upon operator binding. Thus, in the regions of *lac* repressor probed by these substitutions, the inducer-bound form differs from the conformation of the unliganded form.
The roles of His$^{74}$ and Asp$^{278}$ in operator and inducer binding were also explored. His$^{74}$ and Asp$^{278}$ form a potential salt bridge between the N- and C- subdomains in the repressor core. Mutations were generated at both positions to vary size, charge, and polarity in an effort to determine the nature and importance of this interaction. All repressor proteins produced by mutating His$^{74}$ and/or Asp$^{278}$ possess altered ligand binding behavior. Interestingly, only the H74W mutant repressor did not display the characteristic ~1000-fold decrease in operator affinity when bound to inducer.

The oligomeric state of lac repressor is a result of the free energy involved in both assembly and folding. The overall stability of tetrameric repressor was determined by two approaches. The free energy of complete unfolding by urea-induced denaturation of tetrameric repressor is ~49.1 kcal/mol. The total free energy for denaturation of tetrameric repressor calculated by combining all of the assembly and folding events is ~58.8 kcal/mol. These results demonstrate unequivocally that the tetrameric lac repressor is an extremely stable protein. However, the difference between these free energy values suggests that the same transitions may not be monitored in the two different approaches.
Acknowledgments

My education at Rice University has been supported and encouraged by many people. Kathy Matthews has provided me with a tremendous amount of freedom and resources to explore my interests. Not only have I learned from her how to better interpret results and assess their relevance to overall research goals, but she has also given me a better appreciation for the subtleties of dealing with people as individuals. I also appreciate the guidance provided by the members of my thesis committee: Dr. John Olson, Dr. George Bennett, and Dr. Ed Nikonowicz. Their continued enthusiasm for lac repressor will always be appreciated. John Olson has been especially helpful with his encouragement to pursue the small flecks of gold which always appear instead of the nuggets.

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Chapter 1

Introduction

*Lac Operon*

When Jacob and Monod published their operon hypothesis for the regulation of bacterial carbohydrate metabolism, one important component was a soluble regulatory factor (Jacob & Monod, 1961). This factor was identified as a protein that bound to operator DNA and had its binding activity modulated by the presence of specific sugars (Gilbert & Müller-Hill, 1966). The *lac* repressor protein, as the first transcriptional repressor protein to be discovered, has served as a model for how proteins can regulate gene expression in the cell. To function efficiently, the *lac* repressor must be sensitive to the levels of ligands present and must respond quickly. Multiple mechanisms, including allostery and DNA looping, modulate the affinity of *lac* repressor for operator sites to generate tightly controlled gene expression.

The *lac* operon consists of the genes for transporting lactose, converting lactose into its component monosaccharides, glucose and galactose, and for metabolizing thiogalactosides (Jacob & Monod, 1961). Both positive and negative control of gene expression ensures adequate
levels of lactose are present and that glucose is absent before the genes in the \textit{lac} operon are transcribed. These processes are diagrammed in Figure 1. The negative regulatory element is the \textit{lac} repressor protein, and the positive regulatory element is the CAP protein. When CAP is associated with cAMP, the complex binds to a site on the promoter and facilitates RNA polymerase binding and transcription (Miller & Reznikoff, 1980). The amount of cAMP available for CAP to bind is inversely related to the amount of glucose in the cell, via effects of glucose on adenylate cyclase (Kolb \textit{et al.}, 1993). Through this mechanism, the transcription of genes for metabolizing alternative carbons sources is not facilitated when glucose, the primary carbohydrate source, is present.

The \textit{lac} repressor protein exerts negative control over the transcription of the \textit{lac} operon genes based on its affinity for the operator DNA sequence in the promotor region of the operon. This affinity can be altered by carbohydrates present in the bacteria. The DNA sequences for the promoter and the primary operator site overlap in the operon (Gilbert \textit{et al.}, 1975), and in the absence of inducer sugars, the repressor protein and the RNA polymerase compete for their respective binding sites (Majors, 1975; Schlax \textit{et al.}, 1995). There is also evidence that the polymerase and repressor can form a ternary complex at the operator-promoter site which may inhibit both
Figure 1. Control of *lac* operon expression. This figure shows the binding of different proteins to operator sequences depending upon the carbohydrate source present for bacterial metabolism. A. Negative control. When no lactose is present the *lac* repressor protein binds to O₁, and RNA polymerase cannot initiate transcription. B. Positive control. When lactose is present, the repressor binds allolactose and no longer interacts with the O₁ site. This allows the RNA polymerase to initiate transcription. If glucose levels are also low, cAMP will interact with CAP and enhance RNA polymerase binding.
elongation and initiation by the polymerase (Straney & Crothers, 1987; Lee & Goldfarb, 1991). The binding of the natural inducer, β-1,6-allolactose, a by-product of β-galactosidase metabolism of lactose, results in a diminished affinity of lac repressor for operator DNA (Jobe & Bourgeois, 1972; Barkely et al., 1975), and transcription is initiated. Functionally, the lac repressor protein has evolved multiple mechanisms to enhance its repression in the absence of lactose and respond quickly when threshold levels of lactose are present.

Allostery

The second important concept to originate in part from studies of lac repressor is allostery (Monod et al., 1963). Allostery is the ability of one ligand to influence the affinity of another ligand, whether they be similar (homotropic) or different (heterotropic) ligands. The structural basis of allostery is that the protein exists in different conformations, each with a high or low affinity for the ligand (Monod et al., 1965). The model that was proposed for the analysis of this behavior is based on the concerted change of an oligomeric structure from a T-state (taut) to the R-state (relaxed) upon binding an effector molecule. These interactions are described in the model by the number of binding sites, the equilibrium constant between the two unliganded oligomeric conformations, L, and the intrinsic dissociation
constant for each ligand, $K_r$ and $K_i$. The antagonism in the lac repressor system between binding to the DNA and repressing transcription or binding to the inducer and allowing transcription can be described by this model (See Appendix) (O’Gorman et al., 1980b; Daly & Matthews, 1986a).

**Elements of the lac repressor system**

The three main components involved in the negative regulation of the lac operon are the lac repressor protein and the two types of ligands with which it can interact, DNA and β-galactosides. The lac repressor protein is a 150,000 Da homotetramer (Gilbert & Müller-Hill, 1966; Riggs & Bourgeois, 1968; Riggs et al., 1968). The protein can associate with two DNA fragments (O’Gorman et al., 1980a; Culard & Maurizot, 1981; Whitson & Matthews, 1986) and four sugar molecules (Ohshima et al., 1974; Butler et al., 1977; O’Gorman et al., 1980b). How this protein interacts with these ligands is the key to its function as a regulatory protein.

*Lac* repressor has a binding affinity of ~0.1 μM for any sequence of DNA; however, this association is highly dependent on the ionic strength of the solution (Lin & Riggs, 1975 a,b; Kao-Huang et al., 1977; Revzin & von Hippel, 1977; O’Gorman et al., 1980a). The repressor protein interacts with a higher affinity (~1000 fold) to sites with specific operator DNA sequences (Gilbert & Müller-Hill, 1967; Riggs et al., 1968; O’Gorman et al., 1980a).
The difference in lac repressor recognition of non-specific versus specific DNA sequences lies in the types of contacts the protein makes with the DNA. Non-specific binding relies primarily upon ionic interactions (~11 contacts), while specific binding has fewer ionic interactions (~6 contacts) but more apolar interactions (Record et al., 1977; de Haseth et al., 1977; Whitson et al., 1986). Nuclease digestion, methylation, and footprinting experiments determined that repressor protein protects 25-31 base pairs of DNA when associated with the operator sequence (Gilbert & Maxam, 1973; Ogata & Gilbert, 1979; Schmitz & Galas, 1979; Manly & Matthews, 1984). The site size for non-specific DNA interactions with repressor was estimated to be ~12-15 bases (Revzin & von Hippel, 1977; Butler et al., 1977).

The natural inducer sugar for lac repressor is β-1,6-allolactose, which is a derivative of lactose produced by β-galactosidase (Jobe & Bourgeois, 1972). Barkley et al. (1975) have identified a number of sugars for which lac repressor has various affinities, most of them greater than 1 mM (Table 1). The presence of hydroxyls at positions C-3 and C-6 have the greatest effect on galactoside binding affinity (Chakerian et al., 1987). IPTG (isopropyl-1-thio-β-D-galactoside) is the most commonly studied ligand because lac repressor binds with a very high affinity, and cannot be
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*Table from Barkley et al., 1975.*
hydrolyzed by $\beta$-galactosidase.

The identity of the substituent at C1 in the $\beta$-galactosides or the nature of the sugar can alter the stability of the repressor-operator-sugar complex. *Lac* repressor binding to inducer sugars results in a ~1000 fold decrease in operator affinity, while anti-inducers differing only slightly in structure, stabilize the operator-repressor complex ~5-fold (Riggs *et al.*, 1970; Barkley *et al.*, 1975). One sugar, ONPG (O-nitrophenyl-$\beta$-D-galactoside), bound the protein but did not alter operator affinity. Competition assays between inducers and anti-inducers determined that these sugars occupy the same binding site (Barkley *et al.*, 1975; Butler *et al.*, 1977).

The crucial functional element for *lac* repressor's role as a negative regulatory protein is allostery, the binding of one ligand altering the affinity for the other. For *lac* repressor, the binding of inducer results in the same affinity for operator DNA as for non-specific DNA (Müller-Hill *et al.*, 1964; Gilbert & Müller-Hill, 1966, 1967; Riggs & Bourgeois, 1968; Barkley *et al.*, 1975). When associated with operator DNA, inducer binding affinity is decreased ~10-fold (Barkley *et al.*, 1975; Dunaway *et al.*, 1980; O'Gorman *et al.*, 1980b). Association with non-specific DNA does not alter inducer binding affinity of the repressor protein (Lin & Riggs, 1975b). Inducer binding to *lac* repressor is cooperative when the protein is bound to operator,
an arrangement which allows the protein to respond rapidly when threshold levels of inducer are present (O'Gorman et al., 1980b). At high pH, lac repressor displays inducer binding characteristics similar to those when bound to operator DNA: binding is cooperative, and there is a ~10-fold decrease in binding affinity (Ohshima et al., 1974; Friedman et al., 1977; O'Gorman et al., 1980b; Daly and Matthews, 1986a). However, at both high pH and simultaneously bound to operator, the inducer binding affinity of lac repressor is further reduced, a result that indicates these two effects are physically distinct (Daly & Matthews, 1986a).

The ligand binding behavior of lac repressor can be modeled using the Monod-Wyman-Changeux (MWC) allosteric model (See Appendix) (O'Gorman et al., 1980b; Daly & Matthews, 1986a). For the binding data to fit the model, a stoichiometry of two operator binding sites and four inducer binding sites is used (O'Gorman et al., 1980b). The allosteric constant predicted by fitting the data to the MWC model was ~1 for wild type lac repressor at pH 7 and ~10^4 at pH 9 (Daly & Matthews, 1986a). These results predicted that wild type repressor would equally occupy both ligand bound conformations at pH 7, while at pH 9, the protein would exist preferentially in the operator bound form (Daly & Matthews, 1986a). While
these models describe the functional mechanisms for allostery, they provide no information regarding how these changes are accomplished structurally.

**Structure of lac repressor**

Identifying the structural elements of *lac* repressor that participate in ligand binding and how these components are assembled provides a better understanding of the mechanisms by which *lac* repressor regulates transcription. The availability of a model for the *lac* repressor core region and crystal structures of the core domain and the complete *lac* repressor protein (Nichols *et al*., 1993; Friedman *et al*., 1995, Lewis *et al*., 1996) provides the structural framework to interpret the numerous functional studies of *lac* repressor protein. The tetrameric structure of the operator-bound *lac* repressor is shown in Figure 2. The individual monomers are each colored separately. The structure demonstrates that the overall quaternary structure is a “dimer of dimers.” The two dimers are held together primarily at the C-terminus, and only a small (300 Å) region of the core domain makes contact across a deep cleft between the dimers (Friedman *et al*., 1995; Lewis *et al*., 1996). The presence of a deep channel in the *lac* protein had been visualized previously by electron microscopy (Ohshima *et al*., 1975; Zingsheim *et al*., 1977). The location of the DNA binding region at the extreme ends of the molecule had been predicted based
Figure 2. Operator-bound structure of *lac* repressor protein. Structure of the tetrameric *lac* repressor derived from PDB file 1LBG (Lewis *et al.*, 1996). Each monomer is colored either blue, cyan, yellow, or green. The 29 base pair DNA fragment is colored magenta. The N- and C-termini and the N- and C-subdomains of the protein are labeled. The figure was drawn in Ribbons version 2.63 (Carson, 1987).
on powder x-ray diffraction data and low-angle neutron and x-ray scattering studies (Steitz et al., 1974; Charlier et al., 1980; Pilz et al., 1980; McKay et al., 1982), although the V-shape of the tetramer was not anticipated.

The initial studies relating lac repressor structure and function were proteolytic digestion experiments which determined that lac repressor was composed of two domains: the N-terminus and the core (Files & Weber, 1976). The functions of these two proteolytic domains were identified based upon ligand binding studies, homology with other proteins, and extensive phenotypic screens prior to solving the crystal structures (Miller et al., 1979; Kleina & Miller, 1990; Markiewicz et al., 1994). The results of these studies were confirmed by the X-ray crystal structure of lac repressor (Friedman et al., 1995; Lewis et al., 1996; Suckow et al., 1996; Pace et al., 1997). Figure 3 shows where in the amino acid sequence these regions of function are located and how these regions were identified by the different studies. The colors representing these domains are superimposed upon the operator-bound lac repressor structure in Figure 4.

N-terminal domain

Multiple experiments have been used to identify the N-terminus of lac repressor as the region which is primarily responsible for specific and non-specific contact with DNA. A significant number of mutations in the
Figure 3. Relationship of \textit{lac} repressor structure to protease digestion, functional regions, and predicted homology with other proteins.

Protease digestion produces two domains of \textit{lac} repressor (Files & Weber, 1976). Phenotypic screens of mutant repressors have demonstrated how the domains of the repressor participate in different functions (Miller, 1979; Kleina & Miller, 1990; Markiewicz \textit{et al.}, 1994). The N-terminal domain (amino acids 1-58) is responsible for DNA binding. The core domain (amino acids 58-360) consists of a region (amino acids 58-340) which is responsible for inducer binding, allostery, and dimer assembly. The C-terminus of the core domain (amino acid residues 340-360) contains the tetramer assembly determinants. These regions have structures which are homologous with other proteins which display the same function (Brennan & Matthews, 1989; Schumacher \textit{et al.}, 1994; Müller-Hill, 1983; Chakerian \textit{et al.}, 1991).
Figure 4. Domain structure of lac repressor. The structural domains depicted in Figure 3 are superimposed upon the operator-bound lac repressor structure. A single dimer is shown from the tetrameric structure in two different orientations. The colors represent the following areas: red is the N-terminal helix-turn-helix (amino acids 1-50), yellow is the hinge helix (51-58), blue is the core domain (residues 60-340), and cyan is the tetramer assembly region (amino acids 340-360). The green residues in the core domain represent the amino acids which are part of the inducer binding cleft (8 Å from the binding site) (Suckow et al., 1996). The operator DNA is colored magenta.
N-terminus were found to abolish operator binding (Adler et al., 1972; Files & Weber, 1976; Miller, 1979; Kleina & Miller, 1990). The single N-terminal fragments, isolated by mild protease digestion of the repressor protein, displayed similar contacts with operator DNA but did not bind with high affinity (Geisler & Weber, 1977; Jovin et al., 1977; Ogata & Gilbert, 1978). Multiple experiments indicated that two N-termini were necessary for specific and stable binding to operator DNA (Kania & Brown, 1976; Geisler & Weber, 1977). Furthermore, this region was shown to have high homology to the family of helix-turn-helix DNA binding proteins (Brennan & Matthews, 1989). NMR structures of the first sixty amino acids confirmed the helix-turn-helix motif structure was present when bound to the DNA (Kaptein et al., 1985; Chuprina et al., 1993) or in the absence of DNA (Slijper et al., 1996). The purification of lac repressor assembly mutants confirmed these conclusions. The isolated monomeric mutants did not bind to operator (Daly & Matthew, 1986b), but dimer mutants possessed high affinity for operator (Brenowitz et al., 1991a; Chen & Matthews, 1992).

**Hinge helix domain**

The importance of amino acid residues 53-58 for specific binding had been identified by phenotypic screens of lac repressor mutants. In these screens, mutation at these residues resulted in a loss of specific binding with
no loss of non-specific binding (Müller-Hill et al., 1977). In the operator-bound crystal structure, this region formed a helix that was undetectable in the inducer-bound structure because the entire N-terminus is disordered (Lewis et al., 1996). Therefore, it was inferred that this helix did not exist in the absence of operator DNA. The presence of the hinge helix was confirmed by NMR experiments based upon changes in resonances for amino acids 50-58 in the presence of DNA (Spronk et al., 1996). These experiments also demonstrated that, in the absence of operator, the amino acid residues 50-62 were disordered. A hinge helix also appears in the operator-bound structure of the homologous PurR protein, indicating that this binding motif is important for the LacI family of proteins (Schumacher et al., 1994).

The hinge helix contributes to specific recognition of the operator DNA by interacting in the minor groove. In the high resolution PurR structure, the side chains of Leu$^{54}$ insert into the minor groove and act as "leucine levers" to alter the conformation of the DNA in the minor groove (Schumacher et al., 1994). An analogous function is predicted for Leu$^{56}$ in lac repressor upon binding DNA (Lewis et al., 1996). Nagadoi et al. (1995) speculated that the hinge helix contacts may play different roles in PurR and lac repressor DNA binding. The amino acid which makes a specific contact
in the minor groove for PurR is Lys$^{55}$. The homologous residue for lac repressor, Ala$^{57}$, cannot distinguish specific base pairs. Therefore, the minor groove contact for lac repressor may not contribute to specific DNA recognition and the role of the minor groove interactions may be different for these two proteins despite the use of the same overall binding motif (Nagadoi et al., 1995).

Core domain

Mutations in the C-terminal part of the protein were found to disrupt inducer binding and repressor assembly (Schmitz et al., 1976; Miller, 1979; Kleina & Miller, 1990; Markiewicz et al., 1994). The bulk of the core domain possesses high homology to the E. coli periplasmic binding proteins (Müller-Hill, 1983), and the region responsible for inducer binding could be modeled based upon the structures of the periplasmic binding proteins for ribose and arabinose (Nichols et al., 1993). Inducer binding occurs in a cleft formed by the N- and C-subdomains of the core (Figure 5A). The residues responsible for contacts with the sugar molecules had been identified previously by genetic screens, homology and mutagenesis (Miller et al., 1979; Sams et al., 1984; Kleina & Miller, 1990; Markiewicz et al., 1994). These results correlated well with those residues identified in the crystal structures (Friedman et al., 1995; Lewis et al., 1996). Figure 5B shows the
**Figure 5. Inducer-bound structure of lac repressor.** A. The dimeric structure of the inducer-bound lac repressor was derived from PDB file 1LBH (Lewis et al., 1996). The residues involved in the inducer binding cleft are shown in green, and the inducer (IPTG) is colored yellow. B. Close-up of the inducer binding cleft. The side chains of the amino acid residues involved in inducer binding are shown. The cyan colored residues (Ser$^{69}$, Arg$^{101}$, Asp$^{149}$, Arg$^{197}$, and Asp$^{149}$) make hydrogen bond interactions with the inducer. The pink residues (Leu$^{72}$, Ala$^{75}$, Pro$^{76}$, Ile$^{79}$, Trp$^{220}$, Phe$^{293}$, and Leu$^{296}$) make hydrophobic contacts (Friedman et al., 1995; Lewis et al., 1996).
amino acid side chains of those residues involved in inducer binding.

Changes in the fluorescence emission spectra upon inducer binding indicated that Trp$^{220}$ was located in the inducer binding pocket (Laiken et al., 1972; Sommer et al., 1976). Mutation of this residue to tyrosine resulted in a ~10-fold decrease in inducer binding affinity (Sommer et al., 1976; Gardner & Matthews, 1990). Mutations at the Arg$^{197}$ residue resulted in a decrease in inducer binding affinity (Spotts et al., 1991). The substitution of Asp$^{274}$ with any amino acid resulted in no detectable inducer binding (Chang et al., 1994; Chang & Matthews, 1995).

A mechanism for PurR-corepressor binding has been proposed based upon the crystal structures of periplasmic binding proteins (Schumacher et al., 1995). Crystal structures of these proteins which were unable to make conformational changes upon ligand binding demonstrated that the sugar is associated with the N-subdomain of the cleft (Sacks et al., 1989). PurR has a hydrophobic patch of amino acids in the N-subdomain of its cleft, and Schumacher et al. (1995) speculated that these hydrophobic amino acids hold the corepressor in place while the conformational shifts necessary for the other amino acids to bind occur. The hydrophobic residues (Figure 5B, shown in pink) in the lac repressor sugar binding cleft also form a patch
located mainly in the N-subdomain (Friedman et al., 1995; Lewis et al.,
1996).

*Determinants of assembly*

The availability of crystal structures has also allowed a more detailed understanding of the components responsible for *lac* repressor assembly. The tetramerization domain of *lac* repressor protein was identified at the C-terminus by its amino acid sequence homology with leucine heptad repeat proteins (Chakerian et al., 1991). Deletion and point mutations in this region resulted in dimeric mutants (Chakerian et al., 1991; Alberti et al.,
1991; Chen & Matthews, 1992). These dimeric proteins possess all the functional properties of the tetrameric repressor: specific operator binding, inducer binding, and allostery (Brenowitz et al., 1991a; Chen & Matthews,

The amino acids which interact to form the dimer interface have been more difficult to identify. Genetic studies indicated that mutations in the regions of 221-227 and 270-285 resulted in monomeric proteins (Schmitz et
al., 1976). One of these monomeric proteins which resulted from a mutation in this region, Y282D, was purified and characterized (Daly & Matthews,
1986b). Because the periplasmic binding proteins were monomers, the subunit interface was difficult to model (Nichols et al., 1993). It was not
until the crystal structure was solved that the side chains involved in the monomer-monomer assembly could be definitively identified (Friedman et al., 1995; Lewis et al., 1996).

**A functional role for the tetramer**

The dimeric lac repressor possesses all the necessary functions for regulating gene expression (Brenowitz et al., 1991a; Chen and Matthews, 1992). Association to a tetramer presumably results in greater stability for the protein, a concept explored in more detail in this thesis. However, there are also functional advantages for the repressor to be able to interact at two sites on the DNA. Interacting with two sites results in the formation of DNA loops which enhance the repression of operon expression. The ability to associate non-specifically with non-contiguous DNA may also facilitate the ability of the repressor to locate its specific binding site.

The lac operon contains three DNA sites recognized specifically by the lac repressor protein. The primary operator site, O₁, overlaps with the promotor region (Gilbert et al., 1975), and the two other pseudo-operator binding sites, O₂ and O₃, were identified 400 base pairs downstream in the Z gene and 90 base pairs upstream of O₁, respectively (Reznikoff et al., 1974; Gilbert et al., 1975; Gilbert et al., 1976). Experiments in which these sequences were deleted determined that the O₁ site was essential and that the
O₂ and O₃ sites were necessary for maximum repression (Reznikoff et al., 1974; Pfahl et al., 1979). Deletion of either O₂ or O₃ sites resulted in ~2- to 3-fold decreases in repression while the absence of both these sites resulted in > 50-fold decreased repression (Eismann & Müller-Hill, 1990; Oehler et al., 1990). A dimeric lac repressor mutant displayed only ~20-fold repression which is comparable to the degree of repression observed when only O₁ is present (Oehler et al., 1990).

Interaction of the repressor protein with two operator sites should physically result in the formation of DNA loops (Whitson et al., 1986; Whitson et al., 1987; Flashner & Gralla, 1988; Eismann & Müller-Hill, 1990; Oehler et al., 1990). Experiments which altered the spacing and sequence of the operator sites determined that loops which correlated with the presence of pseudo-operator sites were exceptionally stable (Whitson & Matthews, 1986; Hseih et al., 1987). looped structures are used in other systems to enhance the stability of DNA-protein interactions (Matthews, 1992). In vitro, tetrameric lac repressors can bind to two operators and form loops as visualized by electron microscopy (Krämer et al., 1987) and detected by loss of Brownian motion for beads attached to tethered DNA (Finzi & Gelles, 1995).
Depending upon the nature and strength of the tetrameric interface, loops could form in two different ways. Either dimeric lac repressor could bind individually to each site and then associate at the C-terminus to form tetramer, or the tetramer could bind to one site and then interact with a second operator site. Efforts using pressure dissociation to measure the stability of the dimer-dimer interface have produced numbers that indicate lac repressor would be dimeric at concentrations of protein where operator binding is assayed (~1 nM) (Royer et al. 1986; Royer et al., 1990a). However, analysis of looping occupancy (Brenowitz et al., 1991b) and modeling the thermodynamic parameters under multiple operator binding conditions have failed to detect dimeric repressor contribution to the binding events (Levandoski et al., 1996). These results indicate that the tetramer interface must be more stable than estimated previously from the pressure denaturation experiments.

The crystal structure of lac repressor bound to operator demonstrated how looping could be important for regulating transcription of the lac operon. Lewis et al. (1996) modeled the CAP-operator DNA-lac repressor complex. Tetrameric lac repressor interaction with the O₁ and O₃ sites was more stable in the presence of CAP (Hudson and Fried, 1990). It was suggested that the mechanism for this enhanced stability was CAP bending
the DNA in such a way that lac repressor could interact more favorably with the two sites on the DNA. A stable CAP-operator DNA-lac repressor complex may result functionally in greater repression of the operon in the absence of both glucose and lactose. There are perhaps two advantages to CAP, the transcriptional activator, enhancing repression. This complex may provide stronger repression for conditions in the cell when there are low concentrations of both glucose and lactose and it is therefore essential that the bacteria conserve energy (Lewis et al., 1996). This complex may also sequester RNA polymerase at the transcription start site for quick initiation once repression is lifted (Straney & Crothers, 1987).

The ability of lac repressor to locate its target sequence in the genome is enhanced by having two sites that can associate with the DNA. The kinetic rates for lac repressor binding to its specific operator are greater than that for a diffusion limited reaction (Riggs et al., 1970). Two mechanisms are believed to account for the more rapid association rates and also explain how lac repressor efficiently locates the operator site in the bacterial genome (Berg et al., 1981; von Hippel & Berg, 1989). The sliding mechanism involves one dimensional diffusion by the protein along the DNA with association and dissociation steps (Berg et al., 1982). It has been estimated that the lac repressor can stay associated with nonspecific DNA for about
100 base pairs, allowing it to search one dimensionally for its specific binding site (Kao-Haung et al., 1977). The effect of ionic strength on the association rates of longer DNA also indicates that this mechanism may be important for lac repressor-DNA binding (Lin and Riggs, 1975a; Winter & Von Hippel, 1981) The second mechanism involves direct transfer between DNA segments. Interssegment transfer relies on the ability of the protein to interact at two sites with the DNA. In this model, the two DNA binding sites on lac repressor associate with non-contiguous DNA. Therefore, when the protein dissoociates from one DNA binding site, the protein remains associated with the second DNA binding site in a different region of the DNA (Berg et al., 1981; von Hippel & Berg, 1989). The lac tetramer probably uses a combination of both these methods to efficiently locate the operator binding sequences in the cell (Ruusala & Crothers, 1992).

**Conformational changes upon ligand binding**

The structural basis of allostery is that different protein conformations result in different ligand binding affinities. Interaction of the protein with a ligand stabilizes the high affinity conformation for that ligand (Monod et al., 1965). Evidence for ligand-induced changes in the structure of lac repressor has been extensively explored. Based upon the small differences in methylation protection patterns for repressor binding to operator in the
induced and uninduced form, Ogata and Gilbert (1979) concluded that the changes which occurred in the protein upon binding inducer were probably small and numerous.

Changes in the biophysical properties of the repressor protein have been detected in the presence of inducer and operator. Inducer binding altered the UV difference spectra and fluorescence spectra compared to free or operator-bound repressor (Ohshima et al., 1972; Laiken et al., 1972; Matthews et al., 1973; Matthews, 1974). Using analytical ultracentrifugation, an increased sedimentation coefficient in the presence of IPTG was interpreted as a conformational change in the presence of inducer (Ohshima et al., 1972). Temperature jump experiments detected two states in a rapid equilibrium at high pH (Wu et al., 1976). Changes in fluorescence anisotropy using both intrinsic fluorescence and an extrinsic probe occurred upon binding inducer (Bandyopadhyay et al., 1981). These experiments detected greater flexibility for the protein when bound to inducer. These studies indicated that conformational changes did occur; however, they did not indicate the specific regions of the protein which were altered.

Lac repressor was chemically modified with probes to detect changes in the protein upon ligand binding. The \(^{19}\text{F}\) NMR spectra of 3-fluorotyrosine-substituted lac repressor demonstrated that the core region
became more rigid upon inducer binding (Jarema et al., 1981). Binding of inducers and anti-inducers resulted in different changes in the absorbance spectra of lac repressor protein labeled with nitrophenol reporter groups (Sams et al., 1977). Modification of the tryptophans with N-bromosuccinimide was blocked by the presence of IPTG, but not by the anti-inducer, ONPF (O’Gorman & Matthews, 1977a). The fluorescence emission spectra of these modified repressor proteins were also altered by inducer (O’Gorman & Matthews, 1977b). Both ONPF and IPTG altered the $^{19}$F NMR spectra of 5-fluorotryptophan-(Trp$^{220}$), but only IPTG changed the spectra of 3-fluorotyrosine-(Tyr$^{282}$) (Boscelli et al., 1981). The Trp$^{220}$ residue was protected from photooxidation when bound to inducer but less protected when bound to anti-inducer (Spodheim-Maurizot et al., 1985).

One approach to specifically detect what regions of the protein were involved in conformational changes was to determine if changes in chemical modification of specific amino acids occurred upon ligand binding. The results from these studies provided two sets of conclusions. The first conclusion was that modification of residues in the N-terminus resulted in a loss of both specific and non-specific binding as seen in the phenotypic analysis of mutants (Miller et al., 1979; Kleina & Miller, 1990; Markiewicz et al., 1994). Modification by reagents specific for arginine residues
(Whitson & Matthews, 1987), lysine residues (Whitson *et al.*, 1984; Hsieh &
Matthews, 1985), histidine residues (Sams & Matthews, 1988), and tyrosine
residues (Fanning, 1975; Boschelli *et al.*, 1981; Hsieh & Matthews, 1981;
Jarema *et al.*, 1981) resulted in decreases in operator binding.

The second conclusion was that residues in the C-terminus had
differing accessibility to modifying reagents. Of the three cysteine residues
in *lac* repressor, Cys$^{281}$ was usually the least reactive to modifying reagents
(Daly *et al.*, 1986). The modification of Cys$^{107}$ and Cys$^{140}$ could be affected
by the presence of operator or inducer (Yang *et al.*, 1977; Burgum &
Matthews, 1978; Brown & Matthews, 1979 a,b). Differences in Cys$^{140}$ I-
AEDANS labeling between the whole protein and the core domain
demonstrated that this residue was in the N-subdomain near the DNA
binding region (Schneider *et al.*, 1984). The modification of Lys$^{209}$ and
Lys$^{327}$ by trinitrobenzenesulfonate was altered by the presence of inducer
(Whitson *et al.*, 1984). In the presence of inducer, the Lys$^{209}$ residue had a
decrease in modification, while the Lys$^{327}$ residue modification was
enhanced (Whitson *et al.*, 1984). These results indicated that conformational
changes sufficient to alter chemical reactivity occurred in the *lac* repressor
core upon inducer binding.
The amino acids participating in the conformational changes associated with allostery were difficult to determine from phenotypic analysis of mutant repressors or chemical studies. The loss of function detected in mutant screens could result from mis-folded or monomeric protein or alteration of the binding site. Changes detected in chemical modification experiments also could derive from effects on folding or binding site residues of the repressor. The availability of a model for the lac repressor core, and crystal structures of the core domain and the complete lac repressor protein, provided the structural information necessary to interpret how these mutations and modifications altered function (Nichols et al., 1993; Friedman et al., 1995, Lewis et al., 1996). By correlating the functional data with location in the structure, regions responsible for allostery can be deduced and further experiments designed to confirm these conclusions (Suckow et al., 1996, Pace et al., 1997).

The crystal structures of lac repressor identified the residues in the monomer-monomer interface and also the regions of this interface which were altered upon ligand binding. Figure 6A shows the backbone residues of the subunit interface colored yellow, and Figure 6B shows the secondary structural elements which demonstrate the greatest difference between the two ligand bound structures colored pink (Lewis et al., 1996). Due to the
Figure 6. The monomer-monomer subunit interface of lac repressor.

The dimeric inducer-bound lac repressor structure is shown. A. The regions of secondary structure which are involved in the monomer-monomer interface are colored yellow. B. The regions of the monomer-monomer interface involved in the allosteric transition are colored pink. This region of the N-subdomain displays a rotation of 10° between the inducer- and operator-bound structures (Lewis et al., 1996). There are three pairs of amino acids which may form salt bridges in the inducer-bound form: Lys^84-Glu^100, Gln^117-Arg^118, and His^74-Asp^278 (Lewis et al., 1996). These residues are shown on each monomer and are colored cyan. In the operator-bound form, these residues are no longer close enough to interact.
low resolution of the operator bound structure, only large domain
movements could be detected in a comparison of different ligand bound
structures. These structures indicate that the C-subdomain remains
essentially constant between the two monomers and that the N-subdomain
rotates about 10°. In the inducer-bound form, there are contacts between β-
strand B (amino acid residues 93-98) and helix 5 (amino acid residues 74-
89) with β-strand B and helix 6 (amino acid residue 104-115) of the opposite
monomer. The rotation of the N-subdomain causes these regions to move
apart and form other unknown contacts. In the inducer-bound structure,
three salt bridge interactions between Lys^{84}-Glu^{100}, Gln^{117}-Arg^{116}, and His^{74}-
Asp^{278} were detected which would no longer be able to make contact upon
the rotation of the interface. These residues are shown in cyan in Figure 6B.

A functional model of allostery for lac repressor has been proposed
based upon the different liganded structures (Lewis et al., 1996). The twist
which occurs during inducer binding in the N-subdomain would cause the
hinge helices to be mispositioned for minor groove binding. Loss of the
hinge helix contacts and accompanying effects on the major groove binding
by the helix-turn-helix motif would result in a decrease in operator binding
affinity in the inducer-bound conformation. The altered conformation of the
N-subdomain would then be stabilized by the new contacts formed across the subunit interace.

This role of the hinge helices in communicating the ligand-bound state of the protein for specific DNA binding has also been detected for PurR (Schumacher et al., 1995). The PurR unliganded protein shows a greater than 20° rotation in its N-subdomain interface compared to the ligand-bound form. While the DNA binding region of this protein also cannot be located in the structure in the absence of DNA, it is predicted that these motions result in the hinge helices being too far apart to interact effectively with the DNA.

**Protein folding**

The mechanisms for protein folding *in vivo* are not very well understood. The basic forces which result in protein folding and stability include hydrophobic packing, electrostatic interactions, and van der Waals forces (Dill, 1990). Hydrophobic packing results in most apolar amino acid side chains existing in the interior of the protein, while most polar residues are at the protein-solvent interface. Electrostatic and van der Waals forces stabilize tertiary structure through interactions between specific amino acid residues and solvent molecules which minimize the free energy associated with the tightly packed, folded protein (Privalov & Gill, 1989).
Protein folding is believed to follow a sequential pathway where short stretches of local secondary structure form first and then assemble as preformed elements (Jaenicke, 1987). For assembly into larger oligomers, subunit recognition requires that the domains involved in quaternary interactions are folded prior to assembly (Jaenicke, 1987). The final protein structure results from a combination of a stable tertiary or quaternary structure combined with whatever flexibility is necessary to maintain functionality.

Experiments designed to study protein folding frequently involve destabilizing the native fold with the use of chemical denaturants or temperature, and monitoring these transitions either by spectroscopic (circular dichroism spectra or the fluorescence of unique tryptophan residues) or physical probes (binding of a specific ligand or appearance of activity). Careful selection of a probe to monitor the unfolding transition is essential with larger, multi-domain proteins because multiple events may occur which might not be detected by a single probe.

The chemical denaturants urea and guanidium chloride are most commonly used to induce protein unfolding. These compounds denature the protein by disrupting the interactions which stabilize helix and \( \beta \)-sheet contacts. When using chemical denaturants to study protein folding, it is
assumed that the free energy of the folding reaction in the absence of
denaturant can be estimated from the free energy at different denaturant
concentrations using linear extrapolation (Pace et al., 1990). In addition, the
ability to renature the protein from the unfolded state is a necessary
precursor to analyzing these reactions assure accurate assessment of the
thermodynamic process.

Folding of oligomeric proteins involves an association reaction which
is second order and therefore concentration dependent (Neet & Timm,
1994). These reactions are frequently more complex than simple first order
folding transitions because kinetic intermediates tend to occur more readily
when folding and assembly are coupled. However, equilibrium constants
can be determined as long as reaction conditions can be found where these
intermediates do not result in large amounts of irreversible protein
aggregation. Such conditions have been determined for lac repressor dimer
formation, and these reactions have been described previously (Chen &
Matthews, 1994; Li & Matthews, 1995; Nichols & Matthews, 1997).

Objectives of this study

The x-ray crystal structures of the lac repressor have identified
regions involved in conformational changes upon ligand binding. To gain a
better understanding of the dynamic components of the allosteric transition,
unique tryptophans were used to probe changes in the environment of these
regions during ligand binding. The functional roles amino acids play in
either the allosteric transition or in stabilizing the different ligand bound
conformations of the protein were also explored. The His$^{74}$-Asp$^{278}$ pair
seems to be uniquely suited to influence allosteric based on its location
between the N- and C-subdomains and in the inducer binding cleft area.
These studies may provide insights into the mechanisms of allostery used by
lac repressor protein.

While the tetrameric lac repressor may play a functional role in fine
tuning repression, it is not essential for the basic function of lac repressor.
To determine if the tetramer or the dimer is the physiologically relevant
oligomeric structure in the bacteria, the stability of this interface was
measured. Knowing the oligomeric state of the protein will allow
deductions regarding the importance of the tetramer versus the dimer in lac
repressor function in vivo to be better assessed.
Chapter 2

Materials and Methods

Double Stranded DNA Procedures

Competent cells and transformation procedures

The competent cells which were used for producing double stranded DNA were bacterial strains E. coli 71-18 (F’ lacIq Δ(lacZ)M15 proA’B’/Δ (lac-pro AB) thi supE) and DH5-α (F’/endA1 hsdR17(ri- m- k+) supE44 thi-1 recA1 gyrA(NaI) relA1 Δ(lacIZYA-argF)U169 deoR(φ80dlac Δ (lacZ)M15). Cells were grown in 2xYT (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, pH 7.5) for liquid culture. LB media (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.5) with 15 g/l agarose added, was used for agarose plates. Ampicillin was prepared as a stock solution at 50 mg/ml and diluted to 50 µg/ml for a final concentration.

Competent cells were prepared by picking a single colony from a plate and allowing it to grow to saturation overnight at 37° C. A 50 ml culture of 2xYT was inoculated, and the cells were grown to an absorbance OD₆₆₀ = 0.4-0.6. Cells were incubated on ice for 15 min and then centrifuged at 4,000 rpm for 5 min to pellet the cells. The pellet was resuspended in 25
ml of 50 mM CaCl$_2$ at 0° C, 15% glycerol, and allowed to incubate at 0° C for 30 min. The cells were then pelleted again and resuspended in 2 ml of the same buffer. These cells were transformed by incubating 50 μl of cells with 1 μl of dsDNA (1 ng/μl) on ice for 30 min. The mixture was then heat shocked at 42° C for 2 min for 71-18 cells or 1 min for DH5-α cells. The reaction mixture was incubated on ice for 1 min before adding 100 μl of ice cold 2xYT. After incubating the mixture for a minimum of 30 min at 37° C, the mixtures were plated and incubated overnight at 37° C.

**Double stranded DNA preparation and sequencing**

For double stranded DNA preparation, a single colony was picked and grown to saturation overnight in 5 ml of 2xYT. Plasmid DNA was purified using a Wizard Kit (Promega). The procedure entailed pelleting 1.5 ml of cells, resuspending and lysing the cells, and precipitating the protein and genomic DNA out of solution. The precipitated material was spun twice for 5 min at 14,000 rpm, and the supernatant was bound to a resin composed of diatomaceous earth. Aliquots of the DNA were run on a 0.9% agarose gel to determine DNA purity.

Plasmid DNA prepared using the Wizard kit could be sequenced using the T7 Sequenase version 2.0 kit (Amersham). The double stranded DNA was denatured for sequencing by incubating 4 μg in 0.2 N NaOH and 1 mM
EDTA for 30 min at 37° C. The reaction was neutralized by 0.3 M NaOAc and precipitated by ethanol.

**Site Specific Mutagenesis**

Mutations in the lac repressor were generated using the method of Kunkel (1985). This method is based upon the binding of an oligonucleotide containing the sequence for the mutation to a single stranded DNA template with the wild type gene which has uracil bases incorporated for a portion of the thymine bases in dut ung cells. The oligonucleotide with the mutant sequence acts as the primer for replication of the template, and the uracil template which contains the wild type DNA sequence is destroyed during the repair process when the DNA is transformed into dut ung E. coli cells.

Two plasmids were used to generate lac repressor mutants, pAC1 and pJC1 (Chakerian & Matthews, 1991; Chen & Matthews, 1992). The pAC1 plasmid containing the lac repressor gene with the W220Y mutation (Gardner & Matthews, 1990) was used to produce the W201Y/W220Y double mutant. Mutagenesis to create the single tryptophan repressor mutant proteins used the uracil template produced from pAC1 with the W201Y/W220Y mutation. The uracil template produced from the pJC1 plasmid was used to create the single point mutants of the lac repressor protein.
To make the uracil template, plasmids were transformed into CJ236 cells (F' (pCJ105; M13'Cm')/dut1 ung1 thi-1 relA1 spoT1 mcrA). A single colony was grown overnight in 2xYT containing 50 µg/ml ampicillin. To 1 ml of this culture, 50 µl of M13K07 phage was added, and the mixture was allowed to incubate for 15 min at room temperature. This solution was added to 50 ml of 2xYT with 50 µg/ml ampicillin and 15 µg/ml chloramphenicol, and the culture was grown until the cells reached an OD₆₆₀=0.2. At this point, 15 µg/ml kanamycin was added to select for the phage-infected cells, and the culture was grown for another four to six hours at 37°C. The culture was spun at 10,000 rpm to remove the cells, and the supernatant was placed in a 20% PEG/2 M NaCl solution at a ratio of 4:1 and incubated in the cold room overnight. This solution was spun at 10,000 rpm, and the pellet was resuspended in 0.5 ml of 0.3 M NaOAc, 1 mM EDTA. The single stranded DNA was extracted with phenol, precipitated with ethanol, and resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). The purity of the single stranded DNA was assessed on an 0.9% agarose gel.

The mutagenic oligonucleotides were synthesized with the base pair changes designed to alter the wild type sequence to encode the selected mutation. The oligonucleotides were either ordered desalted (Great
American Gene Co.) or were desalted using a Sephadex G-50 column (Biorad). A mixture of the mutagenic oligonucleotide, the uracil template, and annealing buffer was heated at 75°C for two minutes and then allowed to cool to room temperature. The extension reaction used T7 Sequenase DNA polymerase (5 units) and T4 ligase (5 units). This reaction mixture was incubated at 0°C for 5 min, room temperature for 5 min, and at 37°C for 90 min. The mutagenesis reaction mixture was then transformed into *E. coli* 71-18 cells, which were grown overnight on plates at 37°C. The double mutant, H74D/D278H, was produced by ligating the HindIII/ApaI fragment from the *lacI* gene which contains the H74D mutation into the pJC1 plasmid with the D278H mutation. Full sequencing of each mutant *lac I* gene was done at the core facility at the University of Texas Medical School at Houston.

*Purification*

Proteins were expressed in either *E. coli* TB-1 cells (JM83 *hsdR* (r<sub>k</sub>*m<sub>k</sub>*)) or BL-26 cells (*ompT hsdS<sub>B</sub> (r<sub>B</sub>*m<sub>B</sub>*)) *gal dcm lac[F' proABlacI<sup>I<sup>9</sup></sup> ZAM15::Tn10 (Tc<sup>R</sup>)]*) which were cured of the episome that carries the I<sup>9</sup> promoter and the I<sup>9</sup> gene. Cells were grown in 2xYT media with 50 μg/ml ampicillin in either 2 liter flasks in a shaker or in the 20 liter fermentor. The cells were harvested by spinning at 4,000 rpm for 20 min, and the pellets
were resuspended in breaking buffer (0.2 M Tris-HCl, pH 7.6, 0.2 M KCl, 0.01 M Mg(Oac)$_2$, 0.3 mM DTT, 50 μg/ml PMSF). The cells were either lysed by sonication or by adding 40 mg of lysozyme to the resuspended pellet and then freezing it at 20°C.

Protein purification followed the previously described procedure (O’Gorman et al., 1980a) with modifications. The lysis mixture was spun at 10,000 rpm, and the supernatant was fractionated with 37% ammonium sulfate and allowed to incubate at 4°C. The ammonium sulfate mixture was spun at 10,000 rpm, and the pellet was resuspended in 0.08 M potassium phosphate, pH 7.5 buffer. This material was dialyzed overnight at 4°C. The dialyzed fraction containing the lac repressor protein was spun at 15,000 rpm for 30 minutes to remove any precipitate before loading onto a phosphocellulose column equilibrated in 0.08 M potassium phosphate, pH 7.5. For tetrameric lac repressor proteins, the column was washed with the same buffer, followed by 0.12 M potassium phosphate buffer wash, and protein was eluted with a gradient from 0.12 M to 0.3 M potassium phosphate. Dimeric lac repressor mutants, -11 aa, K84L/-11 aa and L349A, were eluted from the phosphocellulose column with 0.12 M potassium phosphate buffer after an extensive 0.08 M potassium phosphate buffer wash. Protein concentrations for mutants were determined using absorbance at 280 nm using wild type lac
repressor as a standard. For the mutant repressors in which an additional aromatic amino acid was added, protein concentrations for mutants were determined by Bradford assay (Biorad) using wild type lac repressor as a standard.

The proteins which were not highly expressed (D278H and H74D/D278H) were purified using an additional DEAE column step. The ammonium sulfate pellet was equilibrated in 0.08 M potassium phosphate, pH 7.5, with 5% glycerol. This material was spun at 15,000 rpm to remove any precipitant and then loaded onto a DEAE column equilibrated in 0.08 M potassium phosphate, pH 7.5. The flow through from this column was then loaded onto a phosphocellulose column, also equilibrated in the same buffer. The protein was eluted in the same manner as the other tetrameric lac repressor mutants.

**Repressor-Operator Binding**

The operator used for binding studies was a 40 base pair double stranded DNA (sequence: 5'-TGTGGTGTGGAATTGTGAGCGGATAAC AATTTCACACAGG-3') ordered from Great American Gene Co. Both the top and bottom strands were purified using a 20% polyacrylamide gel to separate the full length oligonucleotides from the incomplete synthesis products. The oligonucleotides were eluted from the gel by incubation
overnight in TE buffer at 37° C with gentle agitation. The top and bottom strand oligonucleotides were hybridized, and this material was labeled at the 5' end with [³²P] by polynucleotide kinase reaction. A Nick column (Pharmacia) was used to purify the labeled 40 base pair operator from the free nucleotides after the reaction was completed.

Operator binding constants were determined for the proteins using the nitrocellulose filter binding assay (Riggs et al., 1968) modified for use in a 96-well dot blot apparatus (Wong & Lohman, 1993). 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 concentration of labeled operator in the assay was ~5x10⁻¹² M or 2.5x10⁻¹³ M for tight binding mutants (O'Gorman et al., 1980a). The concentration of protein was varied from 5 x 10⁻¹³ M to 1.25 x 10⁻⁹ M tetramer depending upon the affinity of the repressor for operator. The amount of [³²P]-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 concentration. These data were fit to the following equation:

\[ R = \frac{[P]}{(K_d + [P])} \]  

(1)
where $R$ is the relative fraction of bound complexes within each solution calculated by $R = \frac{\text{operator bound}}{\text{operator bound}_{\text{max}}}$, $[P]$ is the protein concentration in tetramer, and $K_d$ is the apparent dissociation constant in tetramer concentration. The maximum value for $R$ was floated during the fitting.

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

*Repressor-Inducer Binding*

*Fluorescence titration assay*

Inducer binding was assessed by monitoring the change in fluorescence emission intensity (Laiken *et al.*, 1972). The protein concentration for all mutants was fixed at $5 \times 10^{-7}$ M monomer, and the IPTG concentration was varied between $1 \times 10^{-8}$ and $1 \times 10^{-2}$ M depending upon the affinity of the repressor protein. Proteins were diluted into 0.01 M Tris, 0.15
M KCl buffer at the specified pH. The fluorescence emission was monitored on an SLM 8100 spectrofluorometer using a 340 nm cut-on filter (O-52) from Cominng with an excitation wavelength of 285 nm (Daly & Matthews, 1986a,b). 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 equation:

\[
R = \frac{[\text{IPTG}]^n}{K_d^n + [\text{IPTG}]^n}
\]  

(2)

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

**IPTG competition**

The mutation of W220 causes a decrease of ~10-30 fold in the affinity of the repressor for inducer (Sommer et al., 1976; Gardner & Matthews, 1990) and removes the fluorescence signal most commonly used to monitor inducer binding (Laiken et al., 1972). Therefore, the relative binding of inducer was monitored by following the decrease in operator binding using a nitrocellulose filter binding assay. For mutants with normal operator affinity, the concentration of operator was 5 x 10^{-12} M, and protein was 1.3 x
$10^9$ M tetramer. Protein concentration was increased to $2.6 \times 10^{-8}$ M for mutants with a 10-fold decrease in operator binding affinity. IPTG concentration was varied over a range that spanned the apparent binding affinity. The operator and protein were preincubated in buffer for 15 minutes prior to addition of varying concentrations of IPTG. Samples were filtered, and counts retained were quantified and normalized as described for the operator binding assay. The amount of IPTG necessary to dissociate 50% of the operator/repressor complexes was determined by fitting the competition curves to the equation:

$$R = \frac{[\text{IPTG}]^n}{(Y^n + [\text{IPTG}]^n)}$$

where $R$ is the fraction of operator bound complexes within each solution calculated by $R=\text{bound}/\text{bound}_{\text{max}}$, $n$ is the "apparent" Hill coefficient, and $Y$ is the concentration of IPTG necessary to compete 50% of the operator/repressor complexes. An $n$ value $>1$ in this case reflects the need for more than one IPTG molecule to cause the lac repressor to dissociate from operator.

*Ammonium sulfate precipitation*

This assay followed the protocol of Bourgeois (1971) with the following modifications. The assay buffer contained 0.1 M Tris-HCl, pH
7.4, and 0.15 M KCl. Protein, ranging in concentration from $1 \times 10^{-6}$ M to $1 \times 10^{-5}$ M, was incubated with 15 mg/ml lysozyme which acted as a co-precipitator, and $5 \times 10^{-7}$ M [$^{14}$C]-IPTG for 5 minutes 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. For each condition, a duplicate sample was prepared with the addition of 1 mM unlabeled IPTG to determine the level of nonspecific retention of [$^{14}$C]-IPTG in the pellet. The difference between the samples corresponds to the specific binding of the radiolabeled sugar.

To determine IPTG binding for proteins incubated at different urea concentrations, the ammonium sulfate assay was modified in the following manner. The protein concentration was constant at 5 μM monomer, and the repressors were preincubated in urea concentrations between 0 to 9 M. These samples were incubated with 15 mg/ml lysozyme and $5 \times 10^{-7}$ M [$^{14}$C]-IPTG for 5 minutes before precipitation with 70% saturated ammonium sulfate. The remainder of the assay was performed as detailed above.
**Fluorescence Spectroscopy Measurements**

**Fluorescence emission scans**

Emission spectra were collected on an SLM 8100 spectrofluorometer. The protein was diluted to a final concentration of $5 \times 10^{-7}$ M tetramer in TMS buffer (0.01 M Tris-HCl, pH 7.4, 0.2 M KCl, 0.01 M MgCl$_2$, 1 mM EDTA, and 0.1 mM DTT). The samples were excited at 295 nm, and emission spectra were collected from 300 to 400 nm. All spectra are uncorrected. These spectra were integrated for each mutant to determine the quanta emitted. The UV absorbance peak at 295 nm was measured on an SLM 3000 diode array spectrophotometer to determine the quanta absorbed. The quantum yield was determined by dividing the quanta emitted by the quanta absorbed. The values for the single tryptophan repressors were normalized to the wild type value for comparison.

**Circular dichroism spectroscopy**

*Lac* repressor mutants were diluted to a concentration of $4 \times 10^{-6}$ 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 0.2 cm pathlength quartz cuvette. Data collected for mutants were compared to the wild type protein spectrum.

**Fluorescence lifetimes**
Fluorescence decay curves for the tryptophans in the mutant repressor proteins were collected on an SLM 48000 MHF phase/modulator frequency domain spectrofluorometer equipped with a 10-W Argon laser (Coherent Inc.) with a deep UV output. The laser beam was modulated over a frequency range of 4-280 MHz by a Pockel's cell. Data were collected simultaneously at multiple frequencies and decomposed by means of Fourier transformation. Samples were collected using an emission cut-on filter of 320 nm or, in a few cases, 340 nm. Proteins were diluted into 0.1 M Tris HCl, pH 7.4 or pH 9.2, 0.1 M KCl to a final concentration of 2 μM monomer. Where ligands were present, samples contained a final concentration of 1 x 10⁻³ M IPTG or 1 x 10⁻⁶ M operator DNA for these studies. The intensity decay data between 4-116 Mhz were analyzed as a Fourier transform in terms of the following multi-exponential decay law:

\[ I(t) = I_o \sum \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \]  

(4)

where \( I_o \) has been set to unity, \( \alpha_i \) and \( \tau_i \) are the normalized pre-exponential factor and decay time associated with fluorescence component \( i \), and \( t \) is time. The data are converted into \( f_i \), the fractional intensity, by the relationship:

\[ f_i = \frac{\alpha_i \tau_i}{\sum \alpha_i \tau_i} \]  

(5)
Data analysis was performed with a nonlinear least squares program from SLM and with the program Globals Unlimited (University of Illinois, Urbana, IL) (Beechem *et al.*, 1989). Values obtained from these two different analytical approaches were similar.

*Fluorescence quenching*

Quenching experiments for *lac* repressor proteins were performed as described previously (Gardner & Matthews, 1990; Chang & Matthews, 1995). Stock solutions of potassium iodide, acrylamide, and thallium acetate were prepared at concentrations of 5 M, 4.2 M, and 5 M, respectively. Potassium iodide was prepared fresh each day in buffer with 1 mM sodium thiosulfate to inhibit production of I$_2$ (Lehrer, 1971). Thallium acetate was dissolved in water, and the acrylamide solution was purchased from National Diagnostic Inc. (Eftink & Ghiron, 1976). Proteins were diluted to a concentration of 5 x 10$^{-7}$ M monomer in 0.01 M Tris-HCl, pH 7.4 or 9.2, 0.15 M KCl for the potassium iodide or acrylamide quenching. Samples for thallium acetate quenching were diluted into 0.05 M Tris-acetate, pH 7.4 or 9.2, 0.15 M potassium acetate to avoid formation of insoluble thallium chloride. Samples with ligand contained a final concentration of 1 x 10$^{-3}$ M IPTG or 5 x10$^{-7}$ M operator. Samples were excited at 285 nm and the intensity monitored at the emission wavelength peak for each mutant.
Aliquots of a stock solution of quenching agent (5 μl per addition) were added until the final concentration of potassium iodide, acrylamide, or thallium acetate was 0.25 M, 0.2 M, or 0.25 M, respectively. Fluorescence intensity correction factors were generated using identical titrations with buffer for the potassium iodide and acrylamide samples. A 5 M potassium acetate stock was used for the thallium acetate experiments. Thallium acetate was insoluble at higher concentrations in pH 9.2, and no data could be collected for this condition.

**Chemical Denaturation**

*Unfolding of protein by urea*

Unfolding of tetrameric *lac* repressor by urea was carried out in 0.1 M K$_2$SO$_4$ and 0.01 M Tris-HCl (pH 7.4) as previously described for dimeric mutants (Chen & Matthews, 1994). 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, 16 samples of a fixed amount of *lac* repressor (0.5 μM to 4 μM monomer) were mixed with the urea stock solution to final urea concentrations between 0 to 6 M. Samples were incubated at room temperature for 2 hr before fluorescence and CD signals were determined.
Fluorescence measurements were performed using SLM 8000 or AB2 spectrofluorometers. The fluorescence intensity was monitored at 336 nm with an excitation wavelength of 285 nm. CD spectra were measured using an Aviv Model 60 DS spectropolarimeter. The spectra were collected over a range of 210-300 nm in a 0.2 cm pathlength cuvette, and the intensity of the signal at 222 nm was used for data analyses.

*Refolding of the protein from urea*

Wild type lac repressor could not be refolded from 6 M urea using the procedure developed for the dimeric lac repressor mutants (Chen & Matthews, 1994). An additional renaturation step involving incubation at an intermediate concentration of urea was necessary. The addition of IPTG at this step increased renaturation yields but was not necessary for refolding. Wild type lac repressor was denatured for 2 hours in 5.25 M urea at 28.5 μM monomer concentration at room temperature. Renaturation was initiated by diluting the sample to 2.62 M urea at 14.25 μM monomer concentration and equilibrating for 2 hours. This material was then further diluted to 4 μM monomer at the final urea concentration and allowed to incubate for 2 hours at room temperature before the signal was recorded. The lowest concentration of urea that could be reached using this dilution scheme was 0.7 M urea.
For the operator binding activity assays, urea could be dialyzed completely from the sample. However, dialysis without prior dilution into low concentrations of urea resulted in precipitation of the protein. These samples were renatured for two hours at 2.5 M urea with 1 mM IPTG present. The sample was then exhaustively dialyzed to remove both the urea and the IPTG. Wild type protein and a wild type protein sample that was treated the same as the renatured sample but not exposed to urea were used as controls. The operator assays were performed as described previously.

* Sedimentation equilibrium *

The molecular weight was determined for wild type, -11 aa, K84L, and K84L/-11 aa by sedimentation equilibrium using a Beckman XL-A ultracentrifuge. The sedimentation equilibrium experiments were conducted in 0.1 M K₂SO₄ 0.01 M Tris-HCl (pH 7.4) buffer. Initially, the proteins were characterized in buffer containing no urea at multiple concentrations (7x10⁻⁷ to 8x10⁻⁶ M monomer) and multiple speeds (10,000 – 16,000 rpm). For the urea experiments, the protein concentration was constant at 4 μM monomer, and multiple speeds were employed for each urea concentration. Samples were loaded into a six-channel epon charcoal-filled equilibrium cell and scanned at 280 nm or 230 nm for the samples at 7x10⁻⁷ M monomer. Equilibrium was presumed to be reached when no difference was detected.
between scans four hours apart. The average time for a run consisting of 
three different speeds was 40 hours.

When the protein is at equilibrium in the ultracentrifuge, the 
concentration gradient is a function of molecular weight. The concentration 
gradient can be analyzed to determine molecular weight using the following 
equation:

\[
c_r = c_{r0} \exp\left[\frac{\omega^2}{2RT}M(1 - \bar{\nu} \rho)(r^2 - r_0^2)\right]
\]  

(6)

where \(c_r\) = concentration at radius position \(r\), \(c_{r0}\) = concentration of the 
monomer at the reference radius \(r_0\), \(\omega\) = angular velocity, \(R\) = gas constant 
\((8.314 \times 10^{-7} \text{ erg/mol K})\), \(T\) = temperature in Kelvin, \(M\) = monomer 
molecular weight apparent, \(\bar{\nu}\) = partial specific volume of the solute, \(\rho\) = 
density of the solvent. Data were fit to this equation using the Beckman 
Optima XL-A Data Analysis package provided. Density was calculated 
based upon the method of Kawahara and Tanford (1966). The partial 
specific volume was calculated as 0.7411 for \(lac\) repressor wild type, 0.7413 
for K84L, 0.7420 for K84L/-11 aa, and 0.7425 for -11 aa, in K\(_2\)SO\(_4\) buffer 
based upon the amino acid composition of the proteins using the method of 
Cohn and Edsall (1943). The data were fit assuming that this value did not 
change as a function of urea for the K84L and K84L/-11 aa proteins.

*Analysis of denaturation data*
Spectroscopic signal was assumed to be proportional to the fraction of the native state present at a specific concentration of urea. The intensity of the signal (Y), was converted to fraction unfolded using the equation:

$$F_u = \frac{(Y - Y_f)}{(Y_f - Y_u)}$$

where $Y_f$ and $Y_u$ are the signals of the folded and unfolded states. The ultracentrifuge data were normalized in a similar manner:

$$F_d = M_t - \left(M_{t, app} \left(\frac{M_d}{M_d, app}\right)\right)$$

where the molecular weight in native buffer was $M_t$ for K84L and $M_d$ for K84L/-11 aa and the molecular weight apparent in different concentrations of urea was $M_{t, app}$ for K84L and $M_{d, app}$ for K84L/-11 aa.

The data for dimeric proteins were fit to a two-state model which assumed that the transition occurs with no stable monomeric intermediate, $D \rightleftharpoons 2U$ where D is the native dimer and U is the unfolded monomer, and $K_u = [U]^2/D$ (Chen & Matthews, 1994).

This relationship can be expressed as:

$$F_u = \frac{\sqrt{K_u^2 + 8KP} - K_u}{4[P]}$$

where [P] is the total protein concentration, K is the equilibrium constant for dissociation and unfolding. The free energy value ($\Delta G$) could be determined
based upon the relationship \( K = \exp(-\Delta G/RT) \). The free energy change for this transition in the absence of denaturant was determined by linear extrapolation using the relationship \( \Delta G = \Delta G^\circ + m[\text{urea}] \), where \( m \) is the dependence of \( \Delta G \) on denaturant concentration. The above equations were used to fit single data sets in Igor Pro. NonLin (Johnson & Frasier, 1986) was used to simultaneously fit data sets at different protein concentrations. The slopes of the baselines were fit in all analyses.

For tetrameric repressor protein unfolding, the unfolding processes were more difficult to describe because multiple assembly events and unfolding were occurring at the same time. All models of the tetrameric protein data involved a fourth order dependence on protein concentration. Therefore, an iterative loop was incorporated into the NonLin fitting routine for providing a numerical solution of the fourth order equations. The data were fit using several models.

The first model was \( T_4 \Leftrightarrow 4U \) where \( T_4 \) is the native tetrameric protein, \( U \) is the unfolded monomer, \([P]\) is the total protein concentration in monomer, and \( K_u = [U]^4/T_4 \). The data were fit to fraction unfolded:

\[
f_u = [U]/[P] \tag{10}
\]

The tetrameric protein unfolding data could also be fit to a second model of \( T_4 \Leftrightarrow I_4 \Leftrightarrow 4U \) where \( I_4 \) is an intermediate of unfolded monomers
still associated at the C-terminus, $K_1 = [I_4]/[T_4]$ and $K_2 = [U]^4/[I_4]$. The data was fit to the fraction unfolded:

$$f_u = \frac{4K_2[U]^4}{[P]} + \frac{[U]}{[P]}$$

(11)
Chapter 3

A Fluorescence Study of Ligand-Induced Conformational Changes in *Lac* Repressor

Introduction

The crystallographic structures of *lac* repressor bound to different ligands may provide information about the final conformations that the protein can assume upon ligand binding (Lewis *et al.*, 1996). However, the dynamic processes and possible intermediate structures involved in the transitions between conformations are not evident from these structures. A full understanding of ligand binding and conformational changes occurring in *lac* repressor requires analysis of these events in solution under controlled reaction conditions. Such conformational changes in proteins can be monitored using tryptophan fluorescence emission. Some proteins contain intrinsic tryptophans conveniently located for these measurements. However, in some cases, the tryptophan residues are too far removed from the region of interest, or the relevant fluorescence signal is lost in the background from other tryptophan residues. Site-directed mutagenesis has aided studies of specific tryptophan residues by allowing removal of native tryptophans and placement of single tryptophans in areas of interest. Single
tryptophan-containing proteins have been used to monitor conformational changes or ligand binding in many proteins, including lac repressor, galactose repressor, lac permease, fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase, yeast 3-phosphoglycerate kinase, and recA protein (Sommer et al., 1976; Gardner & Matthews, 1990; Brown et al., 1994; Weitzman et al., 1995; Wantanabe et al., 1996; Cheung & Mas, 1996; Stole & Bryant, 1994).

The two native tryptophans in lac repressor (residues 201 and 220) have been converted to tyrosine and characterized individually (Sommer et al., 1976; Gardner & Matthews, 1990). However, the native tryptophans in lac repressor are located in the inner apolar region of the C-terminal domain and inducer binding pocket of the protein, respectively, and other areas that may be affected by ligand binding could not be monitored. In the present study, a “tryptophan-less” (Wless) lac repressor was produced to remove the native fluorescence emission of Trp\textsuperscript{201} and Trp\textsuperscript{220}, and single tryptophans were introduced in multiple sites, including the DNA binding region, the monomer-monomer subunit interface, and the inducer binding pocket. These areas are predicted to undergo conformational changes during ligand binding. The regions selected for substitution were identified on the basis of a model of the lac repressor monomer, the purine repressor crystallographic
structure, and the structures of the lac repressor core and intact ligand-bound species (Nichols et al., 1993; Schumacher et al., 1994; Schumacher et al., 1995; Friedman et al., 1995; Lewis et al., 1996) Previously published phenotypic screens of numerous lac repressor mutants were used to discern which amino acids within these regions might be mutated to tryptophan with minimal disruption to protein function (Kleina & Miller, 1990). Steady state, quenching, and time resolved fluorescence properties of these mutant proteins were measured to monitor the effects of inducer and operator binding.

Results

Generation of mutants and protein purification

Single tryptophan substitutions at residues Tyr¹⁷, Leu⁶², His⁷⁴, Glu¹⁰⁰, Gln¹¹⁷, Phe²²⁶, Tyr²⁷³, Phe²⁹³, Lys³²⁵, were generated in a “tryptophan-less” background of the double mutation, W201Y/W220Y (Wless). For each mutant, the entire lacI gene was sequenced to confirm that the three mutated residues were the only alterations in the DNA sequence. All mutant repressors were expressed in E. coli lacking the lacI gene and purified by phosphocellulose column chromatography. The purity was assessed by electrophoresis using 10% SDS-PAGE and visualized by silver staining as shown in Figure 7. E100W/Wless and Q117W/Wless were produced in low
Figure 7. Purity of mutant repressor proteins. Protein purity was examined by electrophoresis on a 10% SDS-polyacrylamide gel and visualized by silver stain. Ten μg of repressor protein was loaded for each lane. Lane 1, wild type; lane 2, W201Y; lane 3, W220Y; lane 4, Y7W/Wless; lane 5, L62W/Wless; lane 6, H74W/Wless; lane 7, E100W/Wless; lane 8, Q117W/Wless; lane 9, F226W/Wless; lane 10, Y273W/Wless; lane 11, F293W/Wless; lane 12, K325W/Wless.
quantities and were not as pure as the remaining mutant repressor proteins. Circular dichroism spectra for all repressors demonstrated the same secondary structure content as wild type repressor within experimental error (data not shown).

*Functional properties of single tryptophan mutants*

*Lac* repressor mutants possessing a single native tryptophan have been created previously (Sommer et al., 1976; Gardner & Matthews, 1990). As anticipated from previous studies, the W220Y mutation had minimal effect on operator binding but lowered the affinity for inducer (Table 2), and the W201Y mutation resulted in a 3-fold reduction in operator affinity but had minimal effect on inducer binding. The Wless mutant exhibits similar binding properties with a 10-fold decrease in inducer affinity and a 3-fold lower affinity for operator (Table 2, Figures 8 & 9). Therefore, the Wless mutation results in a *lac* repressor which binds operator and inducer with high affinity, exhibits inducer sensitivity for operator binding, and presumably undergoes a conformational change similar to the wild type protein upon ligand binding.

Several of the single tryptophans introduced into the Wless background affect operator or inducer binding (Table 2, Figure 8 & 9). Inducer binding was monitored indirectly by the effect of IPTG on operator
Table 2: Ligand Binding Properties of Single Tryptophan Mutants

<table>
<thead>
<tr>
<th>repressor</th>
<th>Kₐ operator binding a x10¹¹ M</th>
<th>[IPTG] for 50% operator dissociation b</th>
<th>pH 7.4 x10⁶ M</th>
<th>pH 9.2 x10⁶ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.6 ± 0.1</td>
<td>3.6 ± 1</td>
<td>78 ± 10</td>
<td></td>
</tr>
<tr>
<td>W201Y</td>
<td>5.2 ± 0.9</td>
<td>1.5 ± 0.4</td>
<td>33 ± 4</td>
<td></td>
</tr>
<tr>
<td>W220Y</td>
<td>3.0 ± 0.6</td>
<td>29 ± 3</td>
<td>522 ± 80</td>
<td></td>
</tr>
<tr>
<td>Wless</td>
<td>5.4 ± 0.6</td>
<td>33 ± 5</td>
<td>363 ± 30</td>
<td></td>
</tr>
<tr>
<td>Y7W/WLess</td>
<td>nb c</td>
<td>nd d</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>L62W/Wless</td>
<td>20 ± 3</td>
<td>23 ± 6</td>
<td>267 ± 30</td>
<td></td>
</tr>
<tr>
<td>H74W/Wless</td>
<td>0.51 ± 0.04</td>
<td>ni e</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td>E100W/Wless</td>
<td>5.2 ± 1</td>
<td>47 ± 9</td>
<td>75 ± 10</td>
<td></td>
</tr>
<tr>
<td>Q117W/Wless</td>
<td>1.6 ± 0.4</td>
<td>445 ± 100</td>
<td>&gt;2000</td>
<td></td>
</tr>
<tr>
<td>F226W/Wless</td>
<td>4.8 ± 0.8</td>
<td>51 ± 10</td>
<td>564 ± 60</td>
<td></td>
</tr>
<tr>
<td>Y273W/Wless</td>
<td>7.0 ± 0.8</td>
<td>11 ± 3</td>
<td>150 ± 20</td>
<td></td>
</tr>
<tr>
<td>F293W/Wless</td>
<td>2.0 ± 0.2</td>
<td>520 ± 100</td>
<td>2000</td>
<td></td>
</tr>
<tr>
<td>K325W/Wless</td>
<td>38 ± 10</td>
<td>17 ± 7</td>
<td>181 ± 70</td>
<td></td>
</tr>
</tbody>
</table>

aThe apparent dissociation constant for 40 base pair operator DNA (~2 x 10⁻¹² M) was measured by nitrocellulose filter binding assay. Each value was determined by fitting at least three curves simultaneously to equation 1 in Materials and Methods. bThe relative affinity of the protein for IPTG was determined by competition of repressor/operator complexes by the addition of IPTG. Each value was generated by fitting at least three curves simultaneously to equation 3 in Materials and Methods. Error values were generated by Igor Pro and represent the standard deviation of the fitted curve. cnb=no binding. dnd=not determined. eNi=no induction.
Figure 8. Operator binding curves for mutant repressor proteins. The concentration of labeled DNA was $\sim 2 \times 10^{-12}$ M, and the tetramer protein concentration was varied as indicated. Assays were performed a minimum of three times, and the curves were generated by simultaneously fitting all the data to equation 1 in Materials and Methods. Relative fractional saturation was determined as the ratio of labeled DNA retained by nitrocellulose filter for a specific concentration of repressor to labeled DNA retained at saturating concentrations of repressor.
Figure 9. IPTG competition for operator-bound repressor. [$^{32}$P]-labeled operator DNA and repressor were mixed at concentrations of $5 \times 10^{-12}$ M operator and $2.6 \times 10^{-9}$ M dimer repressor concentration and incubated at room temperature in buffer of the appropriate pH. The concentrations of IPTG added to the solutions are indicated, and these mixtures were incubated an additional 15 minutes. The samples were filtered and analyzed for retention of labeled DNA as described in Materials and Methods. The assays were performed a minimum of three times, and the curves were generated by simultaneously fitting all the data to equation 3 in Materials and Methods. The closed circles (●) are the assay performed in pH 7.4 buffer, and the open circles (○) are the assay performed in pH 9.2 buffer.
binding (Figure 9). The concentration of inducer required for 50%
dissociation of repressor/operator complex is reported in Table 2. The
H74W/Wless mutant displays a 10-fold increase in affinity for operator and
an apparent loss of inducer binding when measured in this assay. The
L62W/Wless and K325W/Wless mutant proteins exhibit a reduction in
operator affinity compared to the Wless background, and the Y7W/Wless
protein does not recognize operator. The Q117W/Wless and F293W/Wless
proteins cause a >10-fold decrease in apparent affinity for inducer and a
return to wild type operator affinity relative to the Wless background.

Although the specific mechanism may be unrelated, lac repressor wild
type protein at pH 9.2 mimics the functional properties of the operator-
bound conformation (O’Gorman et al., 1980b, Daly & Matthews, 1986a;
Friedman et al., 1977). These properties consist of a 10-fold decrease in
affinity and the appearance of cooperative binding for inducer (Hill
coefficient ~1.5). The operator dissociation assay cannot detect
cooperativity at pH 9.2; however, the decrease in inducer affinity between
pH 7.4 and pH 9.2 can be monitored. The only mutant protein in the Wless
background which failed to show the expected decrease in affinity at pH 9.2
was E100W/Wless (Figure 9, Table 2). The Q117W/Wless
repressor/operator complex was not dissociated at all by IPTG at pH 9.2
whereas the F293W/Wless repressor/operator complex could be dissociated with 0.1 M IPTG at pH 9.2 (Figure 9).

The H74W/Wless protein has perhaps the most interesting effect on function. The relative affinity of this protein for inducer could not be measured by operator dissociation because no effect on operator binding was observed at either pH examined. Even at IPTG concentrations of 800 mM or following extended incubation, no release of bound operator was observed. Therefore, an ammonium sulfate precipitation assay was used to determine if H74W/Wless mutant protein actually binds to inducer. The binding of inducer to wild type protein and mutant repressors with different inducer binding affinities is shown in Figure 10. In this assay, the H74W/Wless protein displayed the same inducer binding as F293W/Wless. However, at the concentration of inducer adequate to dissociate operator from F293W/Wless, no decrease in operator binding was detected for H74W/Wless. The inability of H74W/Wless to respond to inducer binding indicates that this repressor may assume irreversibly the conformation with high affinity for operator and diminished affinity for inducer.

**Fluorescence properties**

Steady-state fluorescence spectra were determined for all single tryptophan repressors. The relative quantum yield and emission wavelength
Figure 10. IPTG binding measured by ammonium sulfate assay.

Binding was measured as described in Bourgeois (1971) 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 (▲), W201Y/W220Y (○), H74W/Wless (□), F293W/Wless (■), D274N with a C-terminal histidine tag (Δ). The D274N mutant of lac repressor displays no inducer binding (Chang & Matthews, 1995).
maximum are given in Table 3. A change in the polarity of the environment in which a tryptophan residue resides can result in a change in the emission wavelength. Mutants were screened for changes in their fluorescence spectrum in pH 9.2 buffer or pH 7.4 buffer in the presence of saturating concentrations of ligand. No changes in any emission spectra were detected in the presence of operator or pH 9.2. However, the fluorescence spectra of a subset of the single tryptophan repressors were affected by inducer as shown in Figure 11. The W201Y mutant displays a 10 nm blue shift in emission wavelength and a slight increase in intensity in the presence of inducer (Sommer et al., 1976; Bandyopadhyay & Wu, 1979; Gardner & Matthews, 1990). Sommer et al. (1976) suggested that this increase in intensity is a result of inducer initiating resolubilization of lac repressor which had aggregated during freeze/thaw conditions. The mutants H74W/Wless, Y273W/Wless, and F293W/Wless all shift to the blue end of the spectrum and show a decrease in intensity in the presence of inducer.

*Time-resolved fluorescence*

The fluorescence lifetime values for the single tryptophan mutants under different conditions are listed in Table 4. The exponential decay for lac repressor wild type and the two “native” single tryptophan mutants were best fit to two lifetimes, as observed previously (Brochon et al., 1977; Royer...
### Table 3: Spectral Properties of Single Tryptophan Mutants

<table>
<thead>
<tr>
<th>repressor</th>
<th>$\Phi^p/\Phi_{wt}$</th>
<th>emission maximum (nm)</th>
<th>+IPTG</th>
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</thead>
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<td>1.00</td>
<td>336</td>
<td>327</td>
</tr>
<tr>
<td>W201Y</td>
<td>0.65</td>
<td>338</td>
<td>329</td>
</tr>
<tr>
<td>W220Y</td>
<td>0.45</td>
<td>325</td>
<td>325</td>
</tr>
<tr>
<td>Y7W/Wless</td>
<td>0.29</td>
<td>336</td>
<td>336</td>
</tr>
<tr>
<td>L62W/Wless</td>
<td>0.27</td>
<td>336</td>
<td>336</td>
</tr>
<tr>
<td>H74W/Wless</td>
<td>0.39</td>
<td>348</td>
<td>343</td>
</tr>
<tr>
<td>E100W/Wless</td>
<td>0.23</td>
<td>335</td>
<td>335</td>
</tr>
<tr>
<td>Q117W/Wless</td>
<td>0.46</td>
<td>334</td>
<td>334</td>
</tr>
<tr>
<td>F226W/Wless</td>
<td>0.28</td>
<td>330</td>
<td>330</td>
</tr>
<tr>
<td>Y273W/Wless</td>
<td>0.46</td>
<td>338</td>
<td>332</td>
</tr>
<tr>
<td>F293W/Wless</td>
<td>0.36</td>
<td>329</td>
<td>325</td>
</tr>
<tr>
<td>K325W/Wless</td>
<td>0.31</td>
<td>337</td>
<td>337</td>
</tr>
</tbody>
</table>

*aProtein concentration was 5 x 10^{-7} M tetramer diluted in TMS buffer, pH 7.4. The samples were excited at 295 nm, and emission maximum was the wavelength with the greatest intensity in the spectrum collected from 300 to 400 nm. The quantum yield was determined as described in Materials and Methods.

*bRelative quantum yield was determined by dividing the mutant repressor quantum yield by wild type quantum yield to determine the quantum yield per monomer repressor.
Figure 11. Fluorescence emission spectra of single tryptophan repressors. The repressor samples were diluted to $5 \times 10^{-7}$ M tetramer in TMS buffer, pH 7.4, and excited at 295 nm. The emission spectra were collected from 300 to 400 nm and are represented by a solid line (——). The inducer-bound samples contain $1 \times 10^{-3}$ M IPTG and are represented by a dashed line (---).
<table>
<thead>
<tr>
<th>Condition</th>
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<th>+IPTG</th>
<th>pH 9.2</th>
</tr>
</thead>
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<td>Wild type</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9.0 ± 0.2</td>
<td>0.88 ± 0.01</td>
<td>7.0 ± 0.2</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td>2.1 ± 0.4</td>
<td>0.12 ± 0.01</td>
<td>1.6 ± 0.3</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>W201Y</td>
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</tr>
<tr>
<td>8.9 ± 0.2</td>
<td>0.92 ± 0.01</td>
<td>7.0 ± 0.8</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>1.6 ± 0.6</td>
<td>0.08 ± 0.01</td>
<td>1.1 ± 0.5</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>W220Y</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6.3 ± 0.4</td>
<td>0.84 ± 0.04</td>
<td>5.7 ± 0.4</td>
<td>0.90 ± 0.04</td>
</tr>
<tr>
<td>1.0 ± 0.4</td>
<td>0.16 ± 0.04</td>
<td>1.1 ± 0.8</td>
<td>0.10 ± 0.04</td>
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<tr>
<td>Y7W/Wless</td>
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<td></td>
<td></td>
</tr>
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<td>0.48 ± 0.02</td>
<td>5.4 ± 0.6</td>
<td>0.50 ± 0.04</td>
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<tr>
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<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>&lt;10 ps</td>
<td>0.10 ± 0.05</td>
<td>&lt;10 ps</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>L62W/Wless</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1 ± 0.6</td>
<td>0.58 ± 0.10</td>
<td>5.8 ± 0.1</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>2.1 ± 0.8</td>
<td>0.28 ± 0.06</td>
<td>2.3 ± 0.8</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>&lt;180 ps</td>
<td>0.10 ± 0.06</td>
<td>&lt;250 ps</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>H74W/Wless</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>8.6 ± 0.3</td>
<td>0.66 ± 0.03</td>
<td>7.7 ± 0.5</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>2.3 ± 0.4</td>
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<td>2.4 ± 0.3</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>&lt;100 ps</td>
<td>0.07 ± 0.03</td>
<td>&lt;100 ps</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>E100W/Wless</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6.6 ± 0.7</td>
<td>0.42 ± 0.03</td>
<td>6.3 ± 0.2</td>
<td>0.42 ± 0.07</td>
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<tr>
<td>2.5 ± 0.9</td>
<td>0.43 ± 0.02</td>
<td>1.8 ± 0.1</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>&lt;350 ps</td>
<td>0.13 ± 0.04</td>
<td>&lt;100 ps</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>Q117W/Wless</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.7 ± 0.5</td>
<td>0.60 ± 0.02</td>
<td>6.5 ± 0.2</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td>2.3 ± 0.7</td>
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<td>0.29 ± 0.02</td>
</tr>
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<td>&lt;190 ps</td>
<td>0.09 ± 0.01</td>
<td>&lt;100 ps</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>F226W/Wless</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5.6 ± 1.0</td>
<td>0.58 ± 0.02</td>
<td>5.0 ± 0.8</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>2.3 ± 0.6</td>
<td>0.30 ± 0.04</td>
<td>1.8 ± 0.2</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>&lt;180 ps</td>
<td>0.10 ± 0.04</td>
<td>&lt;10 ps</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Y273W/Wless</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6.2 ± 0.3</td>
<td>0.76 ± 0.01</td>
<td>4.5 ± 0.40</td>
<td>0.75 ± 0.01</td>
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<td>2.6 ± 0.3</td>
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</tr>
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<td>0.03 ± 0.02</td>
<td>&lt;20 ps</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Y293W/Wless</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5.9 ± 1.2</td>
<td>0.48 ± 0.03</td>
<td>5.8 ± 0.7</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>2.2 ± 0.9</td>
<td>0.35 ± 0.04</td>
<td>1.5 ± 0.1</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>&lt;250 ps</td>
<td>0.12 ± 0.03</td>
<td>&lt;10 ps</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>K325W/Wless</td>
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<td></td>
</tr>
<tr>
<td>6.5 ± 0.5</td>
<td>0.46 ± 0.18</td>
<td>6.5 ± 0.4</td>
<td>0.40 ± 0.06</td>
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<tr>
<td>1.9 ± 0.4</td>
<td>0.31 ± 0.09</td>
<td>1.9 ± 0.06</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>&lt;60 ps</td>
<td>0.22 ± 0.03</td>
<td>&lt;20 ps</td>
<td>0.20 ± 0.06</td>
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</table>
Table 4. Lifetimes of Single Tryptophan Mutants under Various Conditions. *Single curves were analyzed for multiple lifetime components as described in Materials and Methods and values are reported as nanoseconds unless noted. The values are an average of 3-8 determinations, and standard deviations are shown. The repressor concentration was 2 x 10^6 M monomer and, where present, IPTG concentration was 1 x 10^{-3} M. Four lifetime components were required to achieve optimal fits for the mutant repressors in the Whless background; however, the longest lifetime component (>40 ns) was not well determined by the analyses and contributed <7% to the fluorescence intensity; therefore, this component is not reported. The shortest lifetime component was also not well determined and is reported as a value less than the mean value obtained. For computation of \( \tau_{av} \) for determination of \( k_q \) (see Table 5), the longest lifetime component was eliminated from the calculation. Lifetimes were collected for all repressors in the presence of 1 x 10^{-6} M operator DNA (equivalent to the dimer concentration); however, no change in lifetime was detected for any mutant repressor compared to the pH 7.4 values, and these values are not reported. \( \tau \) is the lifetime. \( f \) is the fractional intensity of each lifetime calculated according to equation 4 in Materials and Methods.
et al., 1990b). The observed changes in the lifetime values for wild type and W201Y (W220 present) in the presence of inducer were also consistent with those previously published. The decrease in the lifetime of W220 in the inducer-bound form was attributed to contacts with the sulfur of the inducer molecule (Royer et al., 1990b). All of the single tryptophan mutants were best fit to a model with four lifetimes. The longest and shortest lifetimes were not well-determined. Both H74W/Wless and Y273W/Wless lifetimes decreased in the presence of inducer, as shown in Figure 12. Interestingly, the tryptophan at Phe^{293}, a residue which forms hydrophobic interactions with inducer similar to those formed by Trp^{220} in the crystallographic structure, does not display the long lifetime of Trp^{220}, nor does its lifetime change in the presence of inducer.

Fluorescence quenching

The exposure of the single tryptophan moiety in different conditions for each of the mutant proteins was measured by using neutral, anionic and cationic quenchers. The Stern-Volmer and quenching rate constants were determined for all conditions. These results are summarized in Table 5, and Figure 13 contains the Stern-Volmer plots for those mutant proteins which demonstrate changes in tryptophan exposure in response to ligands or pH changes. The linear Stern-Volmer plots indicated that no static quenching
Figure 12. Phase/modulation fluorescence lifetime data for mutant repressor proteins. Protein concentration was 2 μM monomer in 0.1 M Tris-HCl, pH 7.4, 0.15 M KCl at 20°C. Open symbols are data collected in the presence of 1 x 10^{-3} M IPTG. The solid lines are a fit to a four exponential decay (equation 4) using Globals Unlimited (Beechem et al., 1989) as described in Materials and Methods.
<table>
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<tr>
<th>repressor</th>
<th>condition</th>
<th>iodide</th>
<th>acrylamide</th>
<th>thallium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_{SV} \text{ M}^{-1}$</td>
<td>$k_q \text{ M}^{-1} \text{ s}^{-1}$</td>
<td>$K_{SV} \text{ M}^{-1}$</td>
</tr>
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<td>5.4</td>
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<td>0.05</td>
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</tr>
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<td>5.5</td>
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<td>0.10</td>
<td>5.1</td>
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<td>6.0</td>
</tr>
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<td>0.08</td>
<td>4.6</td>
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<td>pH 9.2</td>
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<td>0.11</td>
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<td>w/DNA</td>
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<td>0.15</td>
<td>8.0</td>
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<td>2.2</td>
<td>0.42</td>
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<td>pH 9.2</td>
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<td>0.47</td>
</tr>
<tr>
<td></td>
<td>w/DNA</td>
<td>nq (0.01)</td>
<td>2.4</td>
<td>0.45</td>
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<td>Y7W/Wless</td>
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<td>0.45</td>
<td>7.8</td>
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<td>w/IPTG</td>
<td>1.4</td>
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<td>w/DNA</td>
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<tr>
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<td>0.29</td>
<td>8.2</td>
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<td>w/DNA</td>
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<td>0.37</td>
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<td>H74W/Wless</td>
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<td>nq (0.02)</td>
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<td>0.96</td>
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Table 5: Quenching Properties of Single Tryptophan Proteins* (continued)

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*The $K_{sv}$ values shown are calculated from the equation $F_0/F=1+K_{sv}[Q]$, where $F_0$ is the fluorescence intensity at the emission peak maximum before addition of quencher, $F$ is the intensity after addition of quencher, $[Q]$ is the concentration of quencher and $K_{sv}$ is the Stern-Volmer constant. The quenching rate, $k_q$, is calculated using the following relationship: $k_q = K_{sv}/\tau_{sv}$. The average lifetime, $\tau_{sv}$, was calculated from $\tau_{sv} = \Sigma f_i \tau_i$ using the lifetime data reported in Table 4. All quenching values are an average of two to four replicates and the standard deviation for all conditions did not exceed 35% of the reported value, with the exception of thallium acetate quenching for wild-type protein, which had a standard deviation of 50%. All conditions that demonstrated a change in quenching as reported in the text were measured at least three times. \(^b\) nq = not quenched. Not quenched was defined as any sample with an iodide quenching constant ≤0.2. Approximate values for these conditions are reported in parentheses.
FIGURE 13. Stern-Volmer plots of single tryptophan proteins with quenching affected by ligands. The change in fluorescence intensity was plotted as a function of quencher concentration. The solid lines represent a linear fit to the data. The different conditions are represented as follows: pH 7.4 (●), pH 9.2 (○), IPTG (■), and operator (▲).
occurred (Eftink & Ghiron, 1981). The iodide and acrylamide quenching of wild type and the native single tryptophan proteins correlated well with previous studies conducted under saturating concentrations of IPTG (Sommer et al., 1976; Bandyopadhyay & Wu, 1979; Gardner & Matthews, 1990).

Iodide can quench only those tryptophans which are located near the surface of the protein due to its large size and negative charge. The single tryptophan mutants Y7W/Wless, L62W/Wless, E100W/Wless, Q117W/Wless, and K325W/Wless have Stern-Volmer constants greater than one and are highly exposed to solvent. The mutants I174W/Wless, F226W/Wless, and W201Y (Trp$^{220}$ present) have intermediate quenching constants with partially exposed tryptophans residues. Tryptophans at sites buried in the structure, Y273W/Wless and W220Y (Trp$^{201}$ present), are not quenched by iodide. In contrast, the low exposure to iodide quenching observed for the F293W/Wless protein is not consistent with its placement in the inducer binding pocket in the crystallographic structure (Friedman et al., 1995; Lewis et al., 1996).

In the presence of saturating ligands, no changes are detected under any conditions for the mutant repressor proteins in which tryptophans are highly exposed to iodide. These residues may be too exposed to act as
conformational probes, and no conclusions about structural or environmental changes in these regions can be drawn. Similarly, measurement of iodide quenching with and without ligands did not detect any change in the exposure of the buried residues; however, iodide may be too large to penetrate and probe these areas. The only ligand to produce a change in the exposure of intermediately buried tryptophans was inducer. In the presence of inducer, H74W/Wless was less exposed to iodide and F293W/Wless was more exposed. As determined previously, both wild type and W201Y are less exposed to iodide in the presence of inducer (Sommer et al., 1976; Gardner & Matthews, 1990).

Acrylamide is a small neutral quencher that has the ability to penetrate the interior of the protein. The results of acrylamide quenching correlate with iodide quenching; exposed mutants have acrylamide Stern-Volmer constants greater than 7, moderately exposed mutants have acrylamide Stern-Volmer constants between 7 and 4, and the buried residues are less than 4. The F293W/Wless protein is moderately exposed to acrylamide in contrast to its low exposure to iodide and demonstrates no change of exposure in the presence of inducer. The wild type, W201Y and H74W/Wless proteins exhibit lower Stern-Volmer constants in the presence of inducer. However, the rate of quenching, $k_q$, does not change due to a
decrease in the lifetime of the tryptophan in the presence of IPTG.

Bandyopadhyay & Wu (1979) reported the same result for wild type and W201Y mutant repressor protein. For Y273W/Wless, the Stern-Volmer constant remains the same in the presence of inducer, but the decrease in the tryptophan lifetime results in an increase in the rate constant for quenching.

Thallium is a large, positively charged quencher that can be used to assess whether inconsistencies between iodide and acrylamide quenching are a result of a charged amino acid in the area. While other quenchers detected no changes in exposure in the presence of ligands for Y273W/Wless, exposure to thallium was decreased in the presence of both operator and inducer. In the presence of inducer, the F293W/Wless protein was less exposed to thallium. Therefore, the iodide and thallium quenchers, which have opposite charges, demonstrate different exposures for the F293W/Wless tryptophan residue. This result indicates that a charged residue may be in the vicinity of this tryptophan. No changes in the exposure of wild type, W201Y, or H74W/Wless were detected by thallium in the presence of inducer.

Discussion

Conformational changes associated with ligand binding
While operator and inducer bind to the lac repressor at separate functional sites within the structure, the affinities for these ligands are nonetheless reciprocally related, presumably via conformational shifts in the protein in different ligand-bound states (Miller & Reznikoff, 1980). These conformational changes associated with ligand binding in lac repressor have been explored previously by determining the accessibility of residues to chemical modification (Yang et al., 1977; Burgum & Matthews, 1978; Hsieh & Matthews, 1981; Whitson et al., 1984), detecting spectral changes in tryptophan and tyrosine residues (Ohshima et al., 1972; Matthews, 1974; Boschelli et al., 1981; Jarema et al., 1981), and other biophysical properties, in particular sedimentation constants (Ohshima et al., 1972). In temperature jump experiments, the presence of IPTG or protons affected the equilibrium of the conformational transition (Wu et al., 1976). Changes in spectral properties and chemical modification of lysines, tryptophans, and cysteines indicated that conformational changes occurred in the core region upon IPTG binding (Yang et al., 1977; Burgum & Matthews, 1977; Whitson et al., 1984; Sams et al., 1977; Brown & Matthews, 1979a,b; Matthews, 1974; O’Gorman & Matthews, 1977a,b). Both specific and nonspecific DNA binding also caused changes in the chemical modification of amino acid side chains and in the spectral properties of the modified residues (Yang et al.,
1977; Burgum & Matthews, 1977; Hsieh & Matthews, 1981; Whitson et al., 1984; Brown & Matthews, 1979a,b; Kelsey et al., 1979). Since binding different ligands altered the pattern of modification or resulted in spectral changes in comparison with the unliganded form, lac repressor was hypothesized to exist in two extreme conformations, with the unliganded form in equilibrium between the operator- and inducer-bound conformations (Daly & Matthews, 1986a; Laiken et al., 1972; Wu et al., 1976). The availability of the crystallographic structures for free protein and both ligand-bound states provides the opportunity to identify regions that may contribute to linking these functions or may be affected by the conformational alterations associated with binding (Friedman et al., 1995; Lewis et al., 1996). Surprisingly, in contrast to the multiple changes in the spectral properties and reactivity of side chains to chemical modification observed upon inducer binding, the free and inducer-bound forms of the protein were found to be very similar in the crystal structure, while the operator-bound form displayed changes in orientation of the N-subdomain of the core region.

*Tryptophan scanning mutagenesis to probe local environment*

The sensitivity of tryptophan fluorescence to the surrounding environment provides a mechanism to detect structural changes occurring in
the presence of different ligands and has led to intense study of the native tryptophans in the lac repressor protein. Placement of Trp$^{201}$ in the interior of the C-subdomain and Trp$^{220}$ in the inducer binding site by genetic and biochemical studies (Laiken et al., 1972; Sommer et al., 1976; Gardner & Matthews, 1990) has been confirmed by the crystallographic structures (Friedman et al., 1995; Lewis et al., 1996). The diminished inducer affinity caused by the W220Y mutation and all mutants containing this alteration can be expected based on the role of this side chain in binding to inducer. By selective substitutions of tryptophan in a Wless background (W201Y/W220Y), different regions of the protein can be monitored specifically and exclusively to explore the dynamic events responsible for the mutual influence of different ligands on functional properties and on the conformation of the lac repressor protein (Figure 14).

*Exposed tryptophan side chains in single mutants unaffected by ligand, while spectral properties of buried residues altered*

The single tryptophan mutants generated in this study can be grouped into two categories: exposed and buried. Based upon the emission wavelength and quenching data, the substitutions of Y7W/Wless,
Figure 14. *Lac* repressor core domain structure with residues substituted by tryptophan. Only one dimer of the tetrameric structure is shown. Structure of the dimer *lac* repressor core region bound to inducer was derived from PDB file 1LBH (Lewis *et al.*, 1996). One monomer is represented in green and the other monomer is colored blue. The residues substituted with tryptophan residues are shown in one of the monomers. The monomer-monomer interface residues (His$^{74}$, Glu$^{100}$, Gln$^{117}$, and Phe$^{226}$) are colored red, the interior residues (Tyr$^{273}$ and Phe$^{293}$) are colored rose, and the exposed residues (Leu$^{62}$ and Lys$^{325}$) are colored gold. The Tyr$^{7}$ residue 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, the N- and C-subdomains, and the leucine heptad repeat sequences of this structure are labeled.
L62W/Wless, E100W/Wless, Q117W/Wless, and K325W/Wless all generate exposed tryptophan residues. These proteins display large Stern-Volmer quenching constants with both iodide and acrylamide and an emission spectrum with its peak at \( \approx 336 \text{ nm} \). Except for Gln\(^{117} \), all these residues were predicted to be exposed based upon inspection of the X-ray crystallographic structures (Friedman \textit{et al.}, 1995; Lewis \textit{et al.}, 1996) and confirmed by analysis of genetic and structural data by Suckow \textit{et al.} (1996). The substitutions that produced proteins with tryptophans not exposed to solvent, H74W/Wless, Y273W/Wless, and F293W/Wless, exhibited altered spectra and changed quenching properties in the presence of inducer. The spectral and functional properties of these single tryptophan repressors will be examined individually.

\textit{No spectral alterations for exposed tryptophans at Glu}\(^{100} \) and Gln\(^{117} \) \textit{despite placement in region of significant structural change}

The most significant rearrangement between the structures of the different liganded states of \textit{lac} repressor results from a rotation of the monomers relative to each other in the monomer-monomer interface of the N-subdomain of the core (see Figure 14). Two residues (Glu\(^{100} \) or Gln\(^{117} \)) present in this N-subdomain interface were mutated to tryptophan in the tryptophan-less background. In the inducer-bound structure, Glu\(^{100} \) is in contact with Lys\(^{84} \) across the dimer interface of its partner monomer (Lewis
et al., 1996). Mutation of Lys$^{84}$ to glutamate or arginine results in a loss of pH sensitivity for inducer binding (Chang et al., 1993), a result also seen with substitution of tryptophan for Glu$^{100}$. Thus, this partner pair appears to be involved in the pH-associated allostery observed for inducer binding. In the inducer-bound form, Gln$^{117}$ makes contact across the interface with Arg$^{118}$ of the partner monomer (Lewis et al., 1996). Either disruption of this contact or introduction of the bulky tryptophan side chain resulted in a decrease in the affinity for inducer. These mutants emphasize the ability of amino acids not in the binding region to affect ligand binding. In this case, mutation of Gln$^{117}$ to tryptophan, which is located further from the inducer binding site, has a greater impact on inducer binding than mutation of Glu$^{100}$.

The tryptophans substituted in this interface were exposed and demonstrated no change in environment in the presence of ligands. Residues in this interface may be exposed due to the necessity for spatial flexibility to produce conformational change in this region. Alternatively, the large tryptophan residue may be extruded toward solvent due to steric constraints at the interface.

Substitutions Y7W, L62W, and K325W affect operator binding without spectral change

The remaining exposed tryptophan substitution mutant proteins, Y7W/Wless, L62W/Wless, and K325W/Wless, all exhibited altered operator
affinity and no change in fluorescence properties upon ligand binding. In
the wild-type protein, Tyr$^7$ is in the DNA binding region as part of the helix-
turn-helix motif. While no contact is made by Tyr$^7$ with operator, this
residue positions Tyr$^{17}$ to make specific operator-protein contacts (Chuprina
et al., 1993). In the wild-type protein, Leu$^{62}$ is located between the operator
binding region and the core domain in the structure (Friedman et al., 1995;
Lewis et al., 1996). This area appears sufficiently flexible for a tryptophan
substitution, but the bulky tryptophan residue possibly perturbs the spacing
of the N-subdomain and DNA binding headpieces with a consequent
decrease in operator affinity. In the wild-type protein, Lys$^{325}$ is in the area
where the C-subdomain links to the leucine heptad repeats of the dimer-
dimer interface. This region is important for maintaining structural integrity
of the C-subdomain, and a reduction in operator affinity may result from
destabilization of the overall monomer structure. Mutants of the adjacent
residue Arg$^{326}$ also resulted in a loss of operator affinity (Li & Matthews,
1995).

*Tryptophan substitution at His$^{74}$ abolishes inducibility and demonstrates
spectral response to inducer binding in this region*

In the crystal structure of wild type lac repressor bound to inducer,
His$^{74}$ is located in the N-subdomain in the monomer-monomer interface and
forms an electrostatic interaction across this interface with Asp$^{278}$ in the C-
subdomain of its partner monomer. In the operator-bound structure of the protein, rearrangement of the monomer-monomer interface disrupts this contact due to the distance between these two residues (Lewis et al., 1996). Placement of a tryptophan at this site decreases inducer affinity, enhances operator affinity, and results in a loss of induction. Whether these results are unique to altering the specific side chain at position 74 or derive from placing a bulky tryptophan in this region is uncertain. No protein production was observed for the D278W/Wless mutation, further suggesting the importance of this interaction. Although the communication between operator and inducer binding sites has been disrupted, the fluorescence properties of H74W/Wless were altered in the presence of inducer. The shift of the fluorescence emission spectra to a shorter wavelength and decreased exposure to iodide indicates a tryptophan at this site is less exposed in the presence of inducer. Thus, introduction of tryptophan at position 74 generates a protein that does not release operator in response to inducer binding, but still displays alterations in environment associated with inducer binding. This region may be crucial in the conformational alterations that accompany inducer binding.
Fluorescence properties of tryptophan at position 273 affected by inducer and operator binding

The residue Tyr	extsuperscript{273} is located near the inducer binding cleft in the C-subdomain but is not directly involved in binding inducer (Friedman et al., 1995; Lewis et al., 1996). However, the tryptophan residue substituted at position 273 has its emission spectra, lifetime, and accessibility to quencher altered in the presence of inducer. In the presence of inducer, the tryptophan at this position is less exposed to thallium but displays an increased exposure to acrylamide quenching. A similar decrease in exposure may occur for iodide quenching, but the very low accessibility of this tryptophan to iodide precludes accurate analysis. A decrease in exposure to thallium quenching was also detected in the presence of operator. Although residue 273 is not located in the operator binding region, the substitution of tryptophan at this position causes a slight decrease in operator affinity. Previous phenotypic analysis of the Tyr	extsuperscript{273} to serine mutation resulted in a loss of inducer binding, and mutation to glutamine resulted in diminished inducer sensitivity (Kleina & Miller, 1990). These results indicate subtle changes in the region of the structure between the inducer binding cleft and the C-subdomain occur in the presence of both inducer and operator.
Fluorescence properties of tryptophan at position 293 altered by inducer binding

In the wild type protein, Phe$^{293}$ is located at the end of the second crossover helix in the N-subdomain. This residue is part of a hydrophobic patch, including Trp$^{220}$, that participates in inducer binding (Friedman et al., 1995; Lewis et al., 1996). The fluorescence emission spectra of the tryptophans at both positions shifts to a shorter wavelength in the presence of inducer, indicating inducer shields them from solvent. However, Trp$^{220}$ and Phe$^{293}$ are located at opposite ends of the inducer binding pocket, and the other fluorescence properties of these tryptophan residues display different responses to inducer binding. Unlike Trp$^{220}$ and the tryptophan substituted for His$^{74}$ or Tyr$^{273}$, there is no lifetime decrease upon inducer binding for the tryptophan at Phe$^{293}$. In addition, the quenching behavior for the tryptophans at 220 and 293 differs in the presence of inducer. Accessibility of quenchers to the tryptophan at Phe$^{293}$ in the presence of inducer depended upon the charge of the large quenching agents. The presence of inducer increases accessibility to iodide and decreases accessibility to thallium. Complexed to inducer, Trp$^{220}$ is less exposed to iodide but displays no change in exposure to thallium quenching. This comparison of the fluorescence properties of Trp$^{220}$ and F293W/Wless indicates that there are charge and steric differences in their locations in the inducer binding pocket.
Tryptophan substituted at position 226 is partially buried and unresponsive to ligand binding

The tryptophan substitution at residue Phe$^{226}$ is the only partially buried tryptophan whose fluorescence properties were not affected by ligand binding. This result is consistent with the C-subdomain of the monomer-monomer interface being generally more immobile during ligand-induced conformational changes (Lewis et al., 1996). In the crystal structure, residue Phe$^{226}$ participates in a hydrophobic patch that preserves the stability of the dimer interface, and the substitution of a tryptophan does not appear to perturb this function.

In conclusion, the results with lac repressor illustrate the different types of information that can be determined using the sensitivity of tryptophan fluorescence to environmental changes. The combination of genetic studies with the availability of multiple crystal structures has led to the development of a model for conformational change that occurs upon ligand binding to lac repressor (Friedman et al., 1995; Lewis et al., 1996; Kleina & Miller, 1990; Suckow et al., 1996). The crystal structures of the lac repressor in free versus operator-bound states display a rotation of the monomers relative to each other in the N-subdomain of the subunit interface. In contrast, no large changes are seen between free and inducer-bound structures. The fluorescence properties of single tryptophan repressor
proteins displayed no significant changes in response to operator binding; however, inducer binding elicited changes in fluorescence lifetime and in accessibility to different quenchers. The fluorescence data therefore demonstrate structural changes between the unliganded protein and the inducer-bound protein which are not readily evident in a comparison of the crystal structures.

Structural shifts that affect completely exposed or buried residues may not elicit sufficient change in the environment to alter the fluorescence properties of the tryptophan. The inability of exposed probes to detect structural rearrangements possibly accounts for E100W/Wless or Q117W/Wless in the N-subdomain interface not detecting any environmental alteration. Thus, the types of changes which can be monitored by tryptophan probes are selective and depend on local environment and solvent accessibility. In the case of the lac repressor, the residues that yielded significant information were in the central region of the protein proximal to the inducer binding site and were influenced almost exclusively by inducer binding.
Chapter 4

Role of Subunit Interface Residues His$^{74}$ and Asp$^{278}$ in Lac Repressor Allostery

Introduction

The crystal structures of the ligand-bound forms of lac repressor have provided structural information regarding regulation of lac repressor affinity for each of the ligands by binding of the alternate ligand (Lewis et al, 1996). The role that different amino acids play either in the transition of the protein between these two structures or in maintaining the different ligand-bound conformations is uncertain. Three pairs of electrostatic bonds in the inducer-bound structure were detected within the lac repressor monomer-monomer interface: Lys$^{84}$-Glu$^{100}$, Gln$^{117}$-Arg$^{118}$ and His$^{74}$-Asp$^{278}$. The His$^{74}$-Asp$^{278}$ interaction is unique in that it involves one amino acid residue in the N-subdomain (His$^{74}$) with its partner in the C-subdomain (Asp$^{278}$) across the monomer-monomer interface. A comparison of the operator- and inducer-bound structures indicates that in the operator-bound structures these residues have moved apart and can no longer interact (Figure 15). Since the C-subdomain spatial relationships
Figure 15. *Lac* repressor structures with His$^{74}$ and Asp$^{278}$ backbone residues highlighted.  
**A.** *Lac* repressor operator-bound structure. Due to the low resolution of this structure (5.4 Å), no amino acid side chain positions could be determined.  
**B.** *Lac* repressor inducer-bound core structure. The N-terminus is not shown because this region of the protein is not resolved in the crystal structure of the repressor-inducer complex. The His$^{74}$ residues are shown in yellow, and the Asp$^{278}$ residues are shown in green as stick figures.
remain constant during the allosteric transition, the His\textsuperscript{74}-Asp\textsuperscript{278} interaction may be important for maintaining the N-subdomain in the inducer-bound conformation. The role of these residues in the operator-bound structure cannot be determined in the absence of a high resolution crystal structure. The extent to which the His\textsuperscript{74}-Asp\textsuperscript{278} pair is involved in stabilizing the protein in the inducer-bound conformation and in \textit{lac} repressor allostery was explored by altering the side chains of these residues by site-directed mutagenesis.

**Results**

*Generation of mutants and protein purification*

Five substitutions at residue His\textsuperscript{74} (Ala, Leu, Asp, Phe and Trp), seven substitutions at Asp\textsuperscript{278} (Ala, Leu, His, Asn, Glu, Lys, and Trp) and the double substitution (H74D/D278H) were made to explore the importance of this amino acid pair in \textit{lac} repressor allostery. The entire \textit{lacI} gene of each mutant was fully sequenced to determine that no other alterations were present. All mutant repressors were expressed in \textit{E. coli} with the genomic \textit{lacI} gene deleted and were purified by phosphocellulose column chromatography. The D278W mutant was not expressed at sufficient levels for purification. The D278H and the H74D/D278H mutants also were
poorly expressed but could be purified with additional DEAE chromatography. Circular dichroism spectra for all repressors demonstrated the same secondary structure content as wild type repressor within experimental error (data not shown). Based on elution behavior from the phosphocellulose column, all mutant proteins were deduced to be tetrameric.

**Operator binding**

All proteins with mutations at His\textsuperscript{74} or Asp\textsuperscript{278} bound to operator sequences specifically as shown in Figures 16 and 17, and the derived equilibrium dissociation constants are listed in Table 6. Mutation of His\textsuperscript{74} to apolar residues altered operator binding with the effect related to the size of the substitution. Substitution with smaller apolar amino acids (Ala and Leu) decreased operator binding, while larger amino acids (Trp and Phe) 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, distinct from either the H74D or the D278H mutant repressors.

In the presence of inducer, all mutants except for the H74W protein exhibited decreased affinity for operator sequences (1.5 x 10\textsuperscript{-8} M to 1.7 x 10\textsuperscript{-7} M) (Table 6; Figure 16). The H74W mutant repressor bound operator with
Table 6: Operator Binding Properties of Mutant Repressors

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<th>+IPTG $^{b}$</th>
</tr>
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</tr>
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<td>H74F</td>
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<td>$3.8 \pm 0.44$</td>
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<tr>
<td>D278A</td>
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<td>17000</td>
</tr>
<tr>
<td>D278L</td>
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<td>nd$^{c}$</td>
</tr>
<tr>
<td>D278N</td>
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<td>10000</td>
</tr>
<tr>
<td>D278H</td>
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<td>1700</td>
</tr>
<tr>
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</tr>
<tr>
<td>D278K</td>
<td>$0.50 \pm 0.06$</td>
<td>3200</td>
</tr>
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</table>

$^{a}$ The equilibrium dissociation constants of the lac repressor for 40 base pair operator DNA ($2 \times 10^{-12}$ or $5 \times 10^{-13}$ M) were measured using nitrocellulose filter binding assays. Each value was determined by fitting at least three curves simultaneously to Equation 1 as described in Materials and Methods. Error values were generated by Igor Pro and represent the standard deviation of the fitted curve. Values are expressed in dimer.

$^{b}$ Equilibrium dissociation constants were measured in the presence of $1 \times 10^{-3}$ M IPTG. Operator concentration for these assays was $\sim 2 \times 10^{-12}$ M.

$^{c}$ nd=not determined.
Figure 16: Operator binding curves for mutant repressor proteins substituted at His$^{74}$ and/or Asp$^{278}$. The concentration of labeled DNA was $2 \times 10^{-12}$ M or $5 \times 10^{-13}$ M depending upon the affinity of the repressor protein, and protein concentration in dimer was varied as indicated. Assays for specific binding (●) were performed a minimum of two times, and the curves were generated by simultaneously fitting all the data to equation 1 in Materials and Methods. Non-specific binding (⊙) was determined in the presence of $1 \times 10^{-3}$ M IPTG. Relative fractional saturation was determined as the ratio of labeled DNA retained by nitrocellulose filter for a specific concentration of repressor to labeled DNA retained at saturating concentrations of repressor.
high affinity even when complexed with inducer. Its affinity for operator in the presence of inducer was decreased only ~7-fold to 3.6 x 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 non-operator DNA sequence was comparable to wild type repressor bound either to the non-operator DNA or to operator DNA in the presence of inducer (Figure 17).

Mutations in the subunit interface that increase the stability of lac repressor proteins have altered binding kinetics for inducer (Chang et al., 1993; Nichols & Matthews, 1997). Therefore, it was possible that the H74W repressor interface was stabilized such that it could no longer make the allosteric transition. The relative stability of H74W protein compared to wild type protein was explored using urea denaturation. The transition of the H74W repressor denaturation curve was shifted to higher concentrations of urea compared to the wild type repressor curve (Figure 18). While the H74W protein was more stable than wild type repressor, this shift was not as pronounced as when Lys^{84} was mutated to leucine or alanine (Chapter 5). Therefore, increased stability of the protein is probably not the cause of the diminished allosteric response to inducer for H74W. However, one
Figure 17. H74W mutant repressor binding to different operator constructs. The affinity of H74W for 40 base pair operator in the absence (▲) and in the presence of IPTG (△) was determined by nitrocellulose filter binding. The affinity of H74W for a 40 base pair non-specific sequence (CS40i) (▼) was also determined. Binding curves for wild type to CS40i (●) and binding to 40 base pair operator in the presence of IPTG (⊗) are also included. The samples that contained IPTG were incubated for greater than 2 hours with 1 x 10^{-3} M IPTG.
Figure 18. Urea denaturation of H74W mutant repressor. Relative stability of H74W (Θ) was compared to wild type protein (●) by monitoring the decrease in fluorescence as a function of urea concentration. Protein concentration for both these repressors was $4 \times 10^{-6}$ M monomer. Samples were excited at 285 nm, and the emission wavelength peak at 345 nm was recorded. The fluorescence intensity was not normalized for these curves.
possibility is that the bulky tryptophan side chain may sterically interfere with the protein movements required for the allosteric transition to occur.

*Inducer binding*

Inducer binding for *lac* repressor mutants was measured by the change in tryptophan fluorescence upon IPTG binding. At neutral pH, the *lac* repressor equilibrium constant for IPTG binding is $1 \times 10^{-6}$ M. However, in the presence of operator the affinity for inducer is decreased 8–fold (Barkley *et al.*, 1975; Dunaway *et al.*, 1980; O’Gorman, *et al.*, 1980b). For wild type, the same ~10-fold decrease is mimicked in pH 9.2 (Ohshima *et al.*, 1974; Friedman *et al.*, 1977; O’Gorman *et al.*, 1980b). The inducer binding affinity of His$^{74}$ and Asp$^{278}$ mutant repressors was assessed at neutral and high pH and in the presence of operator to determine if these processes were affected. The binding curves are shown in Figure 19, and the $K_d$ values derived from the curves are provided in Table 7. At neutral pH, substitutions at His$^{74}$ resulted in slightly tighter or weaker inducer binding depending upon the size of the residue. The smallest amino acid substitution, alanine, resulted in a slightly higher affinity than wild type. Both H74D and H74L repressors displayed essentially wild type inducer binding. A large amino acid substitution at this residue, phenylalanine, resulted in a ~8-fold decrease in inducer affinity. At elevated pH, smaller amino acid substitutions showed
### Table 7: Inducer Binding Properties of Mutant Repressors

<table>
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<tr>
<th>repressor</th>
<th>pH 7.4 $x 10^6$ M</th>
<th>pH 9.2 $x 10^6$ M</th>
<th>operator DNA $x 10^6$ M</th>
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</thead>
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<tr>
<td>wild type</td>
<td>2.5 ± 0.1</td>
<td>16 ± 0.1</td>
<td>17 ± 0.1</td>
</tr>
<tr>
<td>H74A</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.01</td>
<td>3.5 ± 0.02</td>
</tr>
<tr>
<td>H74L</td>
<td>3.6 ± 0.3</td>
<td>4.0 ± 0.02</td>
<td>8.0 ± 0.05</td>
</tr>
<tr>
<td>H74D</td>
<td>2.1 ± 0.05</td>
<td>2.8 ± 0.02</td>
<td>8.9 ± 0.07</td>
</tr>
<tr>
<td>H74F</td>
<td>20 ± 1.6</td>
<td>140 ± 1.1</td>
<td>270 ± 3.1</td>
</tr>
<tr>
<td>H74W</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>H74D/D278H</td>
<td>3.5 ± 0.03</td>
<td>2.1 ± 0.02</td>
<td>7.3 ± 0.08</td>
</tr>
<tr>
<td>D278A</td>
<td>14 ± 0.4</td>
<td>71 ± 0.33</td>
<td>210 ± 1.9</td>
</tr>
<tr>
<td>D278L</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>D278N</td>
<td>13 ± 0.7</td>
<td>72 ± 0.64</td>
<td>130 ± 1.4</td>
</tr>
<tr>
<td>D278H</td>
<td>5 ± 0.3</td>
<td>10 ± 0.08</td>
<td>60 ± 0.4</td>
</tr>
<tr>
<td>D278E</td>
<td>4.2 ± 1.7</td>
<td>18 ± 0.3</td>
<td>38 ± 0.7</td>
</tr>
<tr>
<td>D278K</td>
<td>4.6 ± 0.2</td>
<td>18 ± 0.2</td>
<td>36 ± 0.6</td>
</tr>
</tbody>
</table>

*Equilibrium dissociation constants for IPTG binding were determined using fluorescence titration experiments. Protein concentration was 5 x 10^{-7} M monomer. Values were determined by simultaneously fitting at least two independent data sets to Equation 2 in Materials and Methods.*

*Samples with operator DNA contained 1 x 10^{-6} M of 40 base pair operator fragment.*

*nd= not determined.*
Figure 19. IPTG titration curves for *lac* mutant repressor proteins.
Fluorescence titrations were performed on an SLM 8100 spectrofluorometer as described in Materials and Methods. The concentration of protein was 5 x 10⁻⁷ M monomer in 0.01 M Tris-HCl, 0.15 M KCl buffer at the indicated pH. Samples with DNA contained 1 x 10⁻⁶ M of 40 base pair operator. The different conditions are represented in the following manner: pH 7 (●), pH 9 (⊗), or in the presence of operator (▲). The curves were generated by simultaneously fitting all the data to Equation 2 in Materials and Methods. Between two and four replicates were fit for each assay condition.
no change in inducer affinity, while the H74F repressor displayed a ~7-fold decrease in affinity compared to its affinity in neutral pH. However, in the presence of operator, inducer affinity was reduced for all of the His$^{74}$ mutants relative to their neutral pH affinity. Both H74A and H74L repressors had slightly decreased affinity, while the H74D protein was decreased by ~4-fold. The H74F repressor had a greater relative shift (~13-fold) compared to wild type protein (~7-fold). These results indicate that different amino acid substitutions at this position alter inducer affinity and the effect of operator and high pH on inducer affinity. Because elevated pH significantly alters the inducer affinity of the H74F repressor, the His$^{74}$ residue is clearly not the sole determinant of the elevated pH effect.

All residues substituted for Asp$^{278}$ resulted in a decreased affinity for inducer at neutral pH. The D278A and D278N mutant repressors displayed 5-fold decreases, 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 the substitution at residue 278 in comparison with the His$^{74}$ substitutions. Since all side chain alterations resulted in a decrease in
affinity at elevated pH, the presence of Asp\textsuperscript{278} in the interface is not essential for this allostery effect.

Interestingly, the double mutant repressor, H74D/D278H, did not reestablish 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 \(\sim\)2-fold, a degree comparable to that observed for the smaller substitutions at His\textsuperscript{74}. However, it cannot be established from these data that the double mutant was able to form the salt bridge as in the wild type repressor.

The additional tryptophan in H74W mutant repressor alters the fluorescence emission spectrum of the protein compared to wild type (Chapter 3). The fluorescence emission spectra, with and without IPTG, of the H74W protein in the W201Y/W220Y background (H74W/Wless) are compared in Figure 20. The binding of IPTG to H74W does not result in the same spectral properties as for wild type protein; the emission wavelength is not shifted to the same degree, and a decrease in intensity occurs which is not seen for wild type. The emission wavelength maximum for unliganded
Figure 20. Fluorescence emission scans of repressor proteins in the absence and presence of inducer. Dashed lines indicate $1 \times 10^3$ M IPTG was present. The repressor samples were diluted to $5 \times 10^{-7}$ M dimer in 0.01 M Tris-HCl, pH 7.4, 0.15 M KCl buffer. The different repressor proteins are represented by the following colors: wild type scans are red, H74W scans are green, and the H74W/Wless scans are blue. These curves represent the actual difference between the mutant repressor fluorescence signal and wild type as the photomultiplier voltage was not changed between samples.
wild type repressor is 336 nm, H74W repressor is 339 nm, and H74W/Wless protein is 343 nm. Upon IPTG binding, emission wavelength maxima of the proteins shift to 329 nm, 336 nm, and 338 nm, respectively. The tryptophan at His$^{74}$ influences the fluorescence emission of the native tryptophan at position 220, especially in the presence of inducer.

The fluorescence emission spectra for H74W and H74W/Wless proteins were collected as a function of inducer concentration at both neutral and high pH (Figure 21 & 22). Both H74W and H74W/Wless repressors showed a slight wavelength shift, an increase, and then a decrease in intensity. Because the same decrease in intensity is not detected for wild type repressor at inducer concentrations greater than 1 $\times$ 10$^{-3}$ M, this decrease is not a result of contaminants in the IPTG quenching the fluorescence signal. At high pH, H74W/Wless protein did not display a decrease in fluorescence. However, the affinity for this repressor at high pH is unknown, and IPTG may not be at a sufficiently high enough concentration to saturate the protein. The slight shift in wavelength along with the changes in intensity made it difficult to assess inducer binding affinity for these proteins using the change in fluorescence signal.

Since the fluorescence titration assay could not be used to determine inducer affinity for H74W repressor, an ammonium sulfate precipitation
Figure 21. Fluorescence emission scans as a function of inducer concentration at pH 7. A. H74W emission scans. B. H74W/Wless emission scans. Samples were incubated at the 10 different IPTG concentrations, but for clarity, only those curves which represented trends in the emission spectra are shown. The spectra at different inducer concentrations for both sets of data are represented by the same colors: red is no IPTG, yellow = 5 x 10^{-6} M, orange = 1 x 10^{-4} M, green = 5 x 10^{-4} M, purple = 5 x 10^{-3} M, and blue = 0.01 M.
Figure 22. Fluorescence emission scans as a function of inducer concentration at pH 9. A. H74W emission scans. B. H74W/Wless emission scans. The spectra for different inducer concentrations for both sets of data are represented by the same colors: red is no IPTG, yellow = 1 x 10^6 M, green = 5 x 10^4 M, purple = 1 x 10^3 M, and blue = 0.01 M.
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, this assay 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 23). 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. Since the H74F and H74W repressors behave similarly in the ammonium sulfate precipitation assay, we concluded 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. The amount of precipitation was dependent on both protein and IPTG concentrations. It was not possible to determine if precipitation was unique to binding IPTG or occurred when other sugars occupied the inducer binding site. 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). Therefore, it was assumed that the affinity of D278L for other sugars is also ~100-fold lower. This result meant that the other sugars
Figure 23. IPTG binding measured by ammonium sulfate assay.

Binding was measured as described in Bourgeois (1971) 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 (▲), H74A (◆), and H74F (■). Red lines and symbols represent samples with $1 \times 10^{-6}$ M of 40 base pair operator.
had to be at concentrations higher than their solubility limit to be at saturating levels for binding to D278L. Therefore, it could not be determined whether other sugars could elicit D278L precipitation.

Binding to IPTG could cause the D278L protein to precipitate if its structure was destabilized or if hydrophobic areas were exposed when the inducer binding site was occupied. Urea denaturation was used to determine if the precipitation was a result of the mutant protein being less stable when bound to IPTG. Fluorescence spectra of D278L repressor samples denatured by urea in the presence of IPTG are shifted in comparison to those in the absence of inducer (Figure 24). This shift demonstrates that the protein was bound to IPTG during the assay. The fluorescence intensity as a function of urea was compared to wild type both in the presence and absence of IPTG (Figure 25). The D278L repressor displayed slightly diminished stability compared to wild type in this assay; however, both of the repressors were more stable when bound to IPTG than the unliganded repressor. Therefore, IPTG binding does not decrease the stability of the D278L mutant repressor.

The exposure of hydrophobic regions upon binding IPTG could be another explanation for the precipitation of D278L protein. The binding of 1-anilino-8-naphthalenesulfonate (ANS) can be used to detect hydrophobic
Figure 24. Fluorescence emission spectra of D278L repressor as a function of urea concentration. The solid lines are fluorescence emission spectra for unliganded D278L repressor at different concentrations of urea; the spectra shown in dashed lines are the same except for the presence of 1 mM IPTG. The different colors represent the following concentrations of urea: black is 0 M urea, yellow is 2.4 M urea, pink is 2.8 M urea, green is 3.2 M urea, blue is 3.6 M urea, and red is 6 M urea. The difference in emission wavelength between the unliganded and inducer-bound spectra demonstrates that IPTG is bound to the D278L protein during the assay.
Figure 25. Urea denaturation of D278L mutant repressor. 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. The denaturation of wild type in the absence (●) and the presence of IPTG (⊗) was included as a reference. Protein concentration was 4 x 10^{-6} M monomer, and samples with inducer present were preincubated with 1 x 10^{-3} M IPTG. Samples were excited at 285 nm, and the emission wavelength peak at 345 nm was recorded.
patches because when ANS binds to the protein, its fluorescence signal is enhanced. Previously, four ANS binding sites were identified between the core and N-terminus for lac repressor, and no ANS fluorescence differences were detected in the presence of IPTG (Worah et al., 1978). To try to decrease the precipitation of D278L protein, experiments were carried out in the presence of 1 M urea. Both D278L and wild type repressor protein still bound inducer at this concentration of urea (see Figure 24). The fluorescence emission spectra of ANS binding to both wild type and D278L proteins is shown in Figure 26. Addition of ANS to D278L resulted in a larger increase in ANS fluorescence compared to wild type ANS binding. The binding of IPTG to wild type did not change the fluorescence of ANS; however, IPTG binding to D278L protein reduced ANS fluorescence to that of ANS bound to wild type protein. No definitive conclusions can be drawn from these ANS binding experiments because precipitation was detected in these samples even at low levels of protein and urea. Therefore, the precise cause of D278L protein precipitation upon binding IPTG is still unknown.

**Thermodynamic analysis**

Fully liganded lac repressor tetramer is bound to four inducer molecules and two operator sequences. The two pathways which result in a
Figure 26. ANS fluorescence emission spectra. The fluorescence emission spectra of ANS bound to D278L protein are shown in red and bound to wild type protein are shown in blue. Repressor protein was diluted to $2.7 \times 10^{-5}$ M monomer in 0.01 M Tris-HCl, pH 7.4, and 0.15 M KCl buffer. ANS concentration was $5 \times 10^{-5}$ M. The dashed lines are the ANS fluorescence spectra when bound to the repressor protein in the presence of 1 mM IPTG. Samples were excited at 355 nm, and the fluorescence emission was recorded from 400 to 600 nm. The curves were not corrected for buffer or the presence of unbound ANS.
completely liganded lac repressor protein are shown in Figure 27. The
equilibrium association constants and free energy values for a subset of these
binding events are in Table 8. Thermodynamic principles predict that the
total free energy of binding should be the same for either $\Delta G_1$ or $\Delta G_2$
pathway. However, the operator DNA binding assay measures only binding
of the first operator because of the low operator DNA concentration used for
these measurements. In contrast, conditions for IPTG binding in the
presence of operator DNA results in measurement of inducer binding
constants for protein bound to two operator DNAs. For the analysis
presented, the assumption was made that repressor bound to a single operator
DNA behaved similarly to repressor bound to two operators in terms of
inducer binding parameters.

The ligand binding behavior of the His$^{74}$ and Asp$^{378}$ mutants was
analyzed in terms of these thermodynamic pathways. For these mutant
repressors, alterations in one ligand binding parameter were compensated by
changes in another. The difference in the total free energy between the two
pathways was greater for the mutant repressors than wild type. Depending
upon the mutant, the variation between the two pathways ranged between 1
and 2 kcal/mol. The H74W repressor was not included in this analysis
because the absolute inducer binding affinity could not be determined. This
Figure 27. Thermodynamic circuit for lac repressor-inducer-operator association. The lines represent the two pathways to produce fully liganded lac repressor, $\Delta G_1$ and $\Delta G_2$. The dashed line represents part of the cycle that could not be measured under operator binding assay conditions. The low operator DNA concentration used for this assay only measures the affinity of a single operator binding event. However, the binding of IPTG is measured when the protein is bound to two operator fragments.
\[ \text{AG} = \text{AG} \] (Repressor + inducer + operator + operon)

\[ \text{AG} = \text{AG} \] (Repressor + inducer + operator + operon)

\[ \text{AG} = \text{AG} \] (Repressor + operon + operator + inducer)

[AG] was calculated from $\text{AG} = \text{K}(\text{T})$, where $\text{T} = 298 \text{ K}$.

Operator binding and monomer affinity for inducer binding.

Values were determined by the relationship $K' = 1/K$. These values are reported as dimer affinity for

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Table 8: Summary of Ligand Binding and Thermodynamic Constants for Mutant Repressors

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repressor probably altered the thermodynamic cycle for ligand binding the most. The H74W repressor demonstrates a ~10-fold decrease in inducer binding in the presence of operator and only a ~7-fold decrease in operator affinity in the presence of inducer. In comparison, wild type repressor displays a ~10-fold reduction in inducer binding in the presence of operator and a ~3000-fold decrease for operator when bound to inducer. Therefore, a large substitution in the subunit interface alters the allosteric transition.

*Monod-Wyman-Changeux Model of Allostery*

Traditionally, *lac* repressor allostery has been described using the Monod-Wyman-Changeux model of concerted allosteric transitions between distinct ligand binding conformations of the protein (O’Gorman *et al.*, 1980b; Daly & Matthews, 1986a). This model relates the alterations in ligand binding to changes in L, the allosteric constant of the protein. It was previously determined that wild type repressor has an allosteric constant near unity (O’Gorman *et al.*, 1980b; Daly & Matthews, 1986a). This value indicates that this protein exists in an equilibrium that favors equally the two ligand-bound states. However, to fit binding data using this model, the intrinsic binding constants must be known. Some *lac* repressor mutants have tighter binding constants for either inducer or operator than wild type (Chang *et al.*, 1994, Müller-Hartmann & Müller-Hill, 1996). 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 is unknown. The intrinsic affinity for IPTG can be
determined because monomeric mutants retain this activity with wild type
tetramer kinetic and thermodynamic parameters (Daly & Matthews, 1986b).
However, since the dimer structure is necessary for operator binding, and the
stability of the dimer can alter the apparent equilibrium constant for operator
binding, the intrinsic operator binding constant is more difficult to determine
(Chen & Matthews, 1994). If the tighter binding values for the intrinsic
binding constants exhibited by mutants are used to determine the allosteric
constant for Monod-Wyman-Changeux behavior, the results suggest that
unliganded lac repressor occupies the inducer bound form. This result is
interesting because the unbound wild type crystal structure was most similar
to the inducer-bound structure (Lewis et al., 1996).

Using the Monod-Wyman-Changeux model, inducer binding in the
absence and presence of operator for the His\textsuperscript{74} and Asp\textsuperscript{278} mutants could be
fit to one set of ligand binding parameters varying only the allosteric
constant (See Appendix). Data were fit with the intrinsic binding constants
used previously for wild type repressor rather than the extreme values
observed for mutant proteins and the allosteric constants generated from
these fits are shown in Table 9. If the more extreme set of constants was used, the L values changed; however, the ligand-bound state toward which the allosteric equilibrium was biased for that mutant did not change. The curves that were generated by using these values and the data are shown in Figure 28. These numbers indicate that mutation at His\textsuperscript{74} can result in stabilizing the equilibrium toward either ligand-bound state depending upon the size of the substitution at this residue. The equilibria of the Asp\textsuperscript{278} mutant repressors were all shifted towards the operator bound state to varying degrees.

Discussion

A comparison of the different ligand-bound X-ray crystallographic structures of lac repressor demonstrates a reorientation of the N-subdomain in the monomer-monomer interface between these forms (Lewis et al., 1996). Even though the residues in this region were not located in either ligand binding site, mutations could alter binding for both inducer and operator (Kleina & Miller, 1990; Markiewicz et al., 1994; Suckow et al., 1996; Pace et al., 1997). These results indicated that this region participated in the allosteric transition. The allosteric properties of lac repressor could altered by perturbing the structural transitions between the
<table>
<thead>
<tr>
<th>repressor</th>
<th>allosteric constant (L)(^{b})</th>
<th>error range</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.73</td>
<td>(0.56 - 0.96)</td>
</tr>
<tr>
<td>H74A</td>
<td>0.0006</td>
<td>(5 x 10(^{-4}) - 8 x 10(^{-4}))</td>
</tr>
<tr>
<td>H74D</td>
<td>0.013</td>
<td>(9 x 10(^{-3}) - 2 x 10(^{-2}))</td>
</tr>
<tr>
<td>H74L</td>
<td>0.009</td>
<td>(6 x 10(^{-3}) - 2 x 10(^{-2}))</td>
</tr>
<tr>
<td>H74F</td>
<td>40000</td>
<td>(3 x 10(^{-4}) - 6 x 10(^{-4}))</td>
</tr>
<tr>
<td>H74D/D278H</td>
<td>0.0063</td>
<td>(4 x 10(^{-3}) - 9 x 10(^{-2}))</td>
</tr>
<tr>
<td>D278A</td>
<td>9700</td>
<td>(8 x 10(^{+3}) - 1 x 10(^{+4}))</td>
</tr>
<tr>
<td>D278H</td>
<td>79</td>
<td>(58 - 100)</td>
</tr>
<tr>
<td>D278N</td>
<td>9500</td>
<td>(7 x 10(^{+3}) - 1 x 10(^{+4}))</td>
</tr>
<tr>
<td>D278E</td>
<td>13</td>
<td>(8.6 - 19)</td>
</tr>
<tr>
<td>D278K</td>
<td>25</td>
<td>(17 - 37)</td>
</tr>
</tbody>
</table>

\(^{a}\) Inducer binding curves in the absence and presence of operator were fit to the Monod-Wyman-Changeux model using NonLin (Johnson & Frasier, 1985). 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 x 10^{-5}\ M^{-1}, K_{RO}= 6.3 x 10^{-6}\ M^{-1}, K_{TO}= 1.7 x 10^{-9}\ M^{-1} and c= 0.011 (Daly & Matthews, 1986a).

\(^{b}\) L=I/R
Figure 28: Theoretical curves generated for inducer binding using the Monod-Wyman-Changeux model of allostery. Curves were generated in Igor Pro using the L values from fitting inducer binding to repressor in the absence (●) and presence of operator (⊗) in NonLin (Johnson & Fraiser, 1985).
different allosteric conformations or by affecting the forces which stabilize or destabilize the ligand-bound conformations. The role of the His$^{74}$-Asp$^{278}$ interaction in lac repressor structure and function was explored based on its location in the monomer-monomer interface in a unique position spanning the N- and C-subdomains across the subunit interface. This pair of side chains is therefore positioned to influence the conformational stability of the inducer-bound form. The most interesting result was that the identity of the residue substituted for His$^{74}$ or Asp$^{278}$ resulted in different allosteric properties. If the salt bridge itself were essential for stabilizing or destabilizing the inducer bound conformation, a mutation at either residue that interrupts this interaction should have the same effect on allostery. Because the nature of the substitution at these residues altered ligand binding properties, the individual roles these two residues play in lac repressor allostery appear more important than their direct contact across the monomer-monomer interface.

The nature of the amino acid substituted for residue His$^{74}$ resulted in dramatically different effects on both the ligand binding and allosteric properties of the mutant repressors. The results from the multiple mutations made at this residue indicate that the size of the substitution is important. The larger amino acids, phenylalanine and tryptophan, both caused a
decrease in inducer affinity and higher affinity for operator. The smaller mutations, alanine, leucine, and aspartate, displayed the opposite behavior. The latter three substitutions all had decreases in operator binding and either wild type or increased affinity for inducer. The hydrophobicity of the substitution did not seem to be as crucial, because leucine and phenylalanine substitutions exhibited different binding characteristics. The aspartate mutation, for which the similar charge should have prevented any interaction with Asp$^{278}$, demonstrated the least change in ligand binding. One reason for this observation could be that the size of aspartate is the most similar to histidine. From these data, we conclude that the size of the amino acid at His$^{74}$ is the most important factor for allosteric properties rather than the formation of a salt bridge.

Substitutions at Asp$^{278}$ did not have as severe effects on allostery as mutations at residue His$^{74}$; all Asp$^{278}$ mutant repressors displayed a similar loss of operator binding in the presence of IPTG. The IPTG binding affinity of all substitutions at D278L was reduced by high pH and in the presence of operator compared to their affinity in neutral pH. Therefore, the salt bridge is not necessary for inducer sensitivity to operator or high pH. However, mutations in this region nonetheless altered the ligand binding properties of the repressor. All mutations at this residue resulted in increased operator
binding affinity and reduced inducer binding affinity. The alteration in ligand binding affinity can be explained based upon the allostERIC equilibrium of the protein being shifted toward the operator-bound form. It is impossible to determine if the effect of mutations at Asp\textsuperscript{278} results from altering its role with His\textsuperscript{74} in the subunit interface or derives from properties unique to the Asp\textsuperscript{278} residue. While mutations at the Asp\textsuperscript{278} residue did not abolish allostERIC behavior, an aspartate at this position appears to be important for maintaining wild type ligand binding, in particular diminishing operator affinity. Increased affinity of these mutant repressors for operator sites could affect the ability of the bacterium to respond effectively to lactose availability.

The location of His\textsuperscript{74} and Asp\textsuperscript{278} in the center of the monomer-monomer interface positions both of these residues to affect the allostERIC transition between the ligand bound states. In the N-subdomain of the inducer bound conformation, β-strand B (93-99) and helix 5 (74-90) contact β-strand B and helix 6 (103-117) of the opposite monomer (Lewis et al., 1996). Upon operator binding, these regions move apart, and the side chains presumably form alternative interactions. Phenotypic analysis of mutations demonstrates that amino acid residues in these regions have different roles in allostery. Mutation of amino acids in β-strand A and
helix 5 (residues 63-84) results in a loss of allostery when hydrophobic amino acids are substituted, while residues in β-strand A (residues 95-97) display alterations in ligand binding when the substitutions are polar or charged amino acids (Suckow et al., 1996).

The His$^{74}$ residue is located at the beginning of helix 5 near the inducer binding cleft (Figure 29). 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 amino acids Leu$^{73}$, Ala$^{75}$, Phe$^{76}$, and Ile$^{79}$ which are important for forming hydrophobic contacts with IPTG in the inducer-bound crystal structures (Friedman et al., 1995; Lewis et al., 1996). Therefore, His$^{74}$ is located at the end of the strand which connects the core with the DNA binding region and is also located in a cluster of amino acids which are important for inducer contacts. The location of His$^{74}$ potentially allows it to play a role in stabilizing the proper contacts for inducer binding which could then influence the relative positioning of the DNA binding regions. The location of Asp$^{278}$ in the C-subdomain, which does not change its orientation upon inducer binding, would anchor the His$^{74}$ residue in the proper conformation for high affinity inducer binding. Conversely, binding
Figure 29. Lac repressor structures with residues connecting the N-terminus and core domain highlighted. A. Operator-bound lac repressor structure with the backbone of residues 62-89 (β-sheet A and helix 5) shown in gray. Residues in helix 5 which interact with inducer (Leu\textsuperscript{73}, Ala\textsuperscript{75}, Pro\textsuperscript{76}, and Ile\textsuperscript{79}) have their backbone colored pink. The His\textsuperscript{74} backbone is colored yellow and the Asp\textsuperscript{278} is colored green. B. Inducer-bound lac repressor structure. The same color scheme as above is used. The higher resolution of this structure allows the His\textsuperscript{74} and Asp\textsuperscript{278} side chains to be shown.
of operator might prevent the His\textsuperscript{74}-Asp\textsuperscript{278} contact and result in lower affinity inducer binding.

High resolution structures are available for purine repressor (PurR), another member of the lac I family, in multiple-ligand bound conformations (Schumacher \textit{et al.}, 1994, Schumacher \textit{et al.}, 1995). In contrast to lac repressor, the purine repressor binds operator specifically in the presence of a corepressor molecule, hypoxanthine or guanine. In the operator-holorepressor structure, PurR residues Glu\textsuperscript{70} and Arg\textsuperscript{278} form a salt bridge between the monomer pairs in the N- and C-subdomains which is an interaction homologous to the His\textsuperscript{74}-Asp\textsuperscript{278} pair in lac repressor (Figure 30B). The Glu\textsuperscript{70}-Arg\textsuperscript{278} contact does not form in the open, unliganded conformation of PurR (Figure 30A). The Glu\textsuperscript{70} residue is located between \(\beta\)-strand A (61-66) and helix I (72-88) of the PurR core, and two residues, Tyr\textsuperscript{73} and Phe\textsuperscript{74}, located in helix I are critical for corepressor binding. From analysis of the crystal structures, these residues, along with Trp\textsuperscript{147}, create a solvent accessible hydrophobic patch for the initial binding site of the corepressor. Upon binding the corepressor, Tyr\textsuperscript{73} and Phe\textsuperscript{74} side chains conformations are altered to stabilize the binding of corepressor. The presence of the Glu\textsuperscript{70}-Asp\textsuperscript{278} salt bridge only in the operator-holoprotein complex of PurR indicates that these charged residues in the subunit
Figure 30: PurR structures with Glu\textsuperscript{70} and Arg\textsuperscript{278} residues displayed as ball and stick figures. The Glu\textsuperscript{70} residue is colored yellow and the Arg\textsuperscript{278} residue is colored green. A. PurR unliganded structure (2.2 Å) was derived from PDB file 1DBQ (Schumacher et al., 1995). The N-terminus is not shown because this region of the protein is not resolved in the crystal structure. B. PurR-corepressor-operator bound structure (2.7 Å) was derived from PDB file 1PNR (Schumacher et al., 1994).
interface are important for allostERIC communication between ligand
binding sites.

A high resolution crystal structure also exists for the unbound form
of PurR. In this PurR structure, Glu\textsuperscript{70} and Arg\textsuperscript{278} are separated from each
other and interact with residues within each monomer. It is difficult to
draw comparisons between the unbound PurR structure and the operator-
bound \textit{lac} repressor structure because of differences in how ligands affect
operator binding. In the absence of a small metabolite, \textit{lac} repressor is
bound to operator, while PurR does not interact with its specific DNA
sequence. The 20\degree rotation of the monomer-monomer interface in the N-
terminal subdomain of PurR compared to the 10\degree rotation of \textit{lac} repressor
could result because PurR is not bound to any ligands in this conformation.

\textit{In vitro} characterization of other \textit{lac} repressor subunit interface
residues in the areas involved in the allostERIC transition indicates that
residues in the monomer-monomer interface are important for both the
structure and function of the \textit{lac} repressor protein. However, these mutations
do not necessarily alter the allostERIC equilibrium of the repressor protein.
Mutations of Lys\textsuperscript{84} in helix 5 to glutamate, alanine, and lysine did not alter
operator or inducer ligand binding affinities. However, introduction of
apolar residues at this position enhanced the stability of the interface and
severely altered the inducer binding kinetics but not thermodynamics (Chang 
et al., 1993; Nichols & Matthews, 1997). Another mutation in helix 5, Ser\textsuperscript{77} to leucine, completely disrupted operator binding and resulted in biphasic inducer binding (Chou & Matthews, 1989). Because the latter mutation resulted in such large changes in ligand binding, it is impossible to assess what role this residue plays in allostery.

Substitution of other subunit interface residues in the regions involved in allosteric transitions results in altered inducer and operator binding affinities. Mutation of Ala\textsuperscript{110}, located in helix 6, affected both inducer and operator ligand binding properties depending upon the nature of the substitution (Müller-Hartmann & Müller-Hill, 1996). At this position, lysine substitution resulted in higher inducer affinity and lower operator affinity, while threonine substitution resulted in higher operator affinity and lower inducer affinity. These results were interpreted in the context of different substitutions stabilizing the repressor in either the inducer- or operator-bound conformation. Another residue that has been substituted and the protein characterized \textit{in vitro} with alterations in both operator and inducer binding is Asp\textsuperscript{88} (Chang \textit{et al.}, 1994). Substitution of lysine for Asp\textsuperscript{88}, located in helix 5, resulted in a decrease in inducer binding and an increase in operator binding affinity. Thus, specific residues in the subunit interface exert
significant influence over the allosteric equilibrium of lac repressor that can be altered by amino acid substitutions.

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. According to the crystal structures, the C-subdomain of the monomer-monomer interface is in a fixed orientation in both ligand-bound structures (Lewis et al., 1996). The precipitation of the D278L repressor in the inducer-bound form may indicate that the interface experiences additional constraints in the inducer-bound form. Interestingly, the larger, charged amino acids, such as lysine, glutamate, and histidine were tolerated at this position and did not elicit precipitation in the presence of inducer.

Modification of subunit interface residues not located in regions where structural changes are observed upon ligand binding also have resulted in binding behavior that can be explained by alteration in the allosteric equilibrium of the modified repressor. The modification of Cys\textsuperscript{281} with methyl methanethiosulfonate (MMTS) resulted in a decrease in operator binding affinity (Daly et al., 1986). 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. However, mutations of Cys\textsuperscript{281} to
alanine, serine, phenylalanine, isoleucine, and methionine, did not alter its ligand binding properties (Chakerian and Matthews, 1991). The Cys$^{281}$ residue is located in a region of the interface which remains constant during the allosteric transition, and the basis for the influence of the MMTS modification on allosteric properties is unclear.

If the loss of a putative His$^{74}$-Asp$^{278}$ salt bridge is responsible for the decrease in inducer affinity at high pH, mutation of either of these residues would result in no change for inducer affinity at high pH compared to the affinity of the mutant repressor at neutral pH. However, some specific His$^{74}$ and Asp$^{278}$ 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 represors. The D278 mutant represors all displayed an almost wild type \(~7\)-fold decrease in inducer affinity. All His$^{74}$ substitutions, except for the H74F repressor, displayed no 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.

It is assumed that elevated pH reduces the inducer binding affinity of the repressor via a quaternary conformational change similar to that caused
by binding operator (O’Gorman et al., 1980b). However, the additional
decrease in inducer affinity when the protein is bound to operator at high pH
indicates that these two allosteric processes result from different mechanisms
(Daly & Matthews, 1986a). Mutation of subunit interface residues in either
the N- and C-subdomains reduces the effect elevated pH has on inducer
binding. In the N-subdomain, mutations of Lys$^{84}$ and Asp$^{88}$ resulted in only
2- and 4-fold decreases in inducer binding at high pH. In the C-subdomain,
the size of the substitution at Cys$^{281}$ correlated with alterations in inducer
binding affinity at elevated pH. The small polar mutations, alanine and
serine, resulted in a protein that demonstrated almost wild type behavior
while proteins with larger, apolar mutations, methionine and phenylalanine,
did not display decreased inducer affinity at elevated pH (Chakerian et al.,
1987). These results indicate that multiple factors, such as charge and size of
the residues in the subunit interface, are responsible for the decrease in
inducer binding at high pH.

The characteristics that influence the effect of high pH on inducer
binding also are relevant to the effect the presence of operator has 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.
Even the His$^{74}$ 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, which reduce inducer binding both at high pH and in the presence of operator. However, there seem to be other factors, such as the presence of charged amino acids in the subunit interface, which may only be important for the allosteric effect of protons on inducer binding. It is possible that the H74F repressor restores the effect of high pH by altering the spacing of the interface to compensate for the loss of the imidazole side chain.

The potential salt bridge between the two interface residues, His\textsuperscript{74} and Asp\textsuperscript{278}, does not appear to contribute to the formation or stabilization of either ligand-bound conformation, since the identity of the residue at either site influences the ligand binding properties of the repressor. 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 (Waldburger et al., 1995). 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 His\textsuperscript{74} and Asp\textsuperscript{278} affects the conformational equilibrium of the repressor protein, and their proximity allows two charged residues to exist in the subunit interface.

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 were also found in the PurR structure (Schumacher \textit{et al.}, 1994). The presence of these similar pairs of interacting residues in two members of this family of proteins indicates that they may be important for the allosteric properties required for the function of these regulatory proteins.

In conclusion, the identities of the amino acids at positions His\textsuperscript{74} and Asp\textsuperscript{278} are important for the allosteric behavior of lac repressor protein. At position His\textsuperscript{74}, the nature of the amino acid substitution altered the allosteric equilibrium toward both ligand-bound conformations. All substitutions at position Asp\textsuperscript{278} resulted in repressor proteins whose equilibrium was biased towards the operator-bound conformation. These results also demonstrated that the presence of the salt bridge between the His\textsuperscript{74} and Asp\textsuperscript{278} residues was not essential for allostery in the lac repressor protein.
Chapter 5

Equilibrium Unfolding Studies of Lac Repressor Protein

Introduction

The ability of lac repressor to diminish transcription of the genes for lactose metabolism is modulated by many mechanisms. The ability of the protein to interact with more than one operator site and form DNA loops is important for maximum repression. The presence of these loops is correlated with reduced transcription in the bacteria (Oehler et al., 1990). The stability of DNA loops, and therefore their contribution to repression, is a combination of DNA-protein interaction as well as protein-protein interaction. If the interaction of lac repressor and DNA is stronger than the tetramer association, at lower concentrations lac repressor would remain bound to DNA but dissociate into dimers which could not form loops.

The lac repressor quaternary structure is very stable, and dissociation into dimers and monomers can only be achieved by mutagenesis (Chen & Matthews, 1994). Because wild type, tetrameric lac repressor does not dissociate at protein concentrations which can be monitored spectroscopically, the overall stability of the wild type protein was determined by urea denaturation. This technique was also used to
determine the strength of the dimer-dimer interface by using a \textit{lac} repressor mutant with a stabilized monomer-monomer interface (Nichols & Matthews, 1997).

Urea denaturation has been used previously to determine the strength of the monomer-monomer interface in a dimeric \textit{lac} repressor mutant (Chen & Matthews, 1994). Figure 31 shows the dimeric \textit{lac} repressor structure with the dimer-dimer interface at the C-terminus colored cyan. Deletion of the last eleven amino acids of this interface results in a dimeric mutant repressor (-11 aa) (Chen & Matthews, 1992). The Lys$^{84}$ amino acid residue in the monomer-monomer interface is shown in pink. Substitution of this residue by leucine or alanine increases the stability of this interface and allows the tetramer to dimer dissociation to be monitored (Nichols & Matthews, 1997).

**Results**

\textit{Wild type lac repressor denaturation}

Tetrameric wild type \textit{lac} repressor was unfolded using the chemical denaturant urea. Figure 32 demonstrates the effect of urea on circular dichroism (CD) and fluorescence emission spectra. Increasing urea concentration causes a decrease in fluorescence intensity and a loss of CD signal intensity. Changes in fluorescence intensity were monitored as a
Figure 31. *Lac* repressor structure with the dimer-dimer interface and residue Lys$^{84}$ highlighted. The complete N-terminus is not shown because this region was not resolved in this crystal structure. The amino acids of the C-terminus which comprise the dimer-dimer interface (amino acids 340-360) are colored cyan. The side chain of the Lys$^{84}$ amino acid residue is represented as a stick figure and colored pink.
Figure 32. Fluorescence and CD spectra of wild type repressor protein at varying concentrations of urea. The protein concentration for all scans was 4 μM monomer. A. Fluorescence emission spectra. B. Circular dichroism spectra. The various concentrations of urea are represented by the following colors: 0 M urea is black, 2 M urea is blue, 3 M urea is red, 3.5 M urea is yellow, 5 M urea is light blue, and 6 M urea is pink.
decrease in signal at 340 nm, and changes in CD signal were monitored as a decrease in the absorbance at 222 nm. The decreases observed for both CD and fluorescence as a function of urea concentration overlap, suggesting that the same transition is being monitored by both spectroscopic techniques (Figure 33). The small deviation between fluorescence and CD at higher urea concentrations, near the end of the transition, may represent residual helical content in the protein. Previously, a dimeric lac repressor mutant was used to establish that the urea denaturation end point of the CD and fluorescence transitions was unfolded monomeric lac repressor (Chen & Matthews, 1994). In Figure 34, the concentration dependence of wild type protein unfolding demonstrates that an assembly process is also being monitored by these spectroscopic probes.

The decrease in fluorescence and CD signal intensity with increasing urea concentration monitors the loss of secondary and tertiary structure of the repressor protein. Information about the oligomeric state of the protein must be determined by other techniques. Equilibrium sedimentation experiments utilizing different centrifuge speeds and protein concentrations demonstrate that lac repressor sediments with a molecular weight of 150,000 Da (Figure 35). In these experiments, wild type protein was tetrameric at concentrations of urea in the regions of the pretransition baseline for the fluorescence and CD experiments. However, after 2.5 M urea, reproducible scans could not be collected in the ultracentrifuge
Figure 33. Denaturation of wild type repressor protein. The protein concentration in this assay was 4 μM monomer diluted into 0.1 M K₂SO₄, 0.01 M Tris-HCl (pH 7.4) buffer with various concentrations of urea. A. Fluorescence intensity (●) and CD (□) signal at 340 nm and 222 nm respectively. B. Normalized fluorescence intensity and CD signal for the data points in panel A. The fraction unfolded, F_u, was determined as described in Equation 7 in Materials and Methods. The wild type unfolding curve at 4 μM monomer was generated from a global fit to the collective data shown in Figure 44.
Figure 34. Concentration dependence of wild type repressor unfolding. Fluorescence intensity was monitored as a function of urea at four different monomeric concentrations of wild type repressor, 4 μM (Δ), 2 μM (▲), 1 μM (○), and 0.5 μM (●).
Figure 35. Equilibrium sedimentation of wild type repressor. Curves were generated by fitting multiple speeds (10,000, 12,000, and 16,000 rpm) and concentrations (2 μM, 4 μM, 8 μM, and 10 μM monomer) to a molecular weight of 150,000 Da using a nonassociating model. For clarity, only the scans representing the following data are shown: 8 μM at 10,000 rpm (●), 8 μM at 12,000 rpm (⊙), and 4 μM at 16,000 rpm (□). The residuals of the data versus the fitted values are shown for each condition.
experiments. Ultracentrifuge data also could not be collected for the -11 aa repressor protein at concentrations of urea beyond the fluorescence and CD pretransition baselines. Therefore, the process causing this variation is not due to the presence of the dimer-dimer interface.

Reversible denaturation of wild type lac repressor

In contrast to the refolding of the dimeric lac repressor mutants, the renaturation of wild type repressor from high concentrations of urea results in significant precipitation and low yields of protein (Schnarr & Maurizot, 1981). When wild type repressor was refolded from 6 M urea in the same manner as -11 aa protein, more than 70% of the protein precipitated at urea concentrations less than 1.5 M. Since dimeric repressors are generated by deleting amino acids at the C-terminus of the protein, the precipitation of the wild type protein upon refolding could result from the additional hydrophobic amino acids at the C-terminus. To determine if wild type protein precipitated because of the full length C-terminus, a lac repressor protein with a single mutation of Leu$^{349}$ to alanine (Chakerian et al., 1991) was unfolded in urea and renatured. This mutation disrupts the leucine heptad repeat sequence and results in a dimeric repressor protein which still possesses the full length amino acid sequence. This protein displayed the same unfolding and refolding behavior as the -11 aa repressor mutant (Figure 36). Therefore, the presence of a non-associating hydrophobic region does not interfere with
Figure 36. Reversibility of L349A mutant repressor denaturation. Urea denaturation (●) and renaturation (○) of 1 μM L349A protein monitored by fluorescence intensity. L349A protein was fully denatured in 6 M urea before dilution to lower urea concentrations for renaturation. Data were normalized as described in Materials and Methods.
lac repressor refolding. These results indicate that the aggregation upon refolding of wild type lac repressor results from the presence of a second protein-protein interface.

A step-wise method of refolding resulted in regeneration of > 80% of the original fluorescence signal (Figure 37). In this procedure, the repressor is incubated at intermediate concentrations of denaturant (2.5 to 3 M urea) before dilution to the final indicated concentration of urea. The addition of IPTG to this step enhanced renaturation yields but was not necessary for refolding to occur. The structural and functional characteristics of the refolded protein were tested to determine whether the restored spectroscopic signal correlates with properly folded, wild type lac repressor. Sedimentation equilibrium experiments using analytical ultracentrifugation demonstrated that the refolded protein was tetrameric with a molecular weight apparent of the 150,000 Da. Figure 38 shows the sedimentation equilibrium data for the renatured and wild type protein were similar.

Specific recognition of DNA requires proper formation of the monomer-monomer interface to align the N-termini in the correct position to contact the operator sites. Changes in protein structure that destabilize or misalign this interface result in a lower operator binding affinity (Chakerian & Matthews, 1991; Chang et al., 1993). Therefore, operator binding activity of the refolded protein was assessed to determine whether
Figure 37. Reversibility of wild type repressor denaturation. Final protein concentration in these assays was 4 μM monomer. A. Fluorescence signal as a function of urea for denatured (●) and renatured (○) wild type repressor. Wild type protein was renatured by incubating samples diluted from 5.25 M urea to 3 M urea prior to dilution to lower concentrations of urea. B. Normalized fluorescence for renatured and denatured wild type repressors.
Figure 38. Sedimentation equilibrium scans of refolded wild type repressor. Wild type protein was renatured as described in Materials and Methods. The control (○) and renatured (●) wild type repressor protein were centrifuged at multiple speeds (12,000 rpm, 14,000 rpm, and 16,000 rpm) at a single concentration of 4 μM monomer until equilibrium was reached. These data sets for the control and renatured samples were each independently fit to a molecular weight of 150,000 Da in a nonassociating model. For clarity, only the scans for one rotor position of the 12,000 rpm data are shown for each sample. The residuals for the data set compared to the fitted values are also included.
a functional monomer-monomer interface is present. Figure 39
demonstrates that the renatured protein bound operator specifically, with
no loss of affinity compared to wild type controls. These experiments in
concert demonstrate that the refolded protein is tetrameric and has a
correctly folded monomer-monomer interface.

Unfolding of a strengthened monomer-monomer interface mutant
repressor

Wild type protein displayed only one transition when monitored by
fluorescence and CD spectroscopy even though multiple processes
(tetramer to dimer dissociation, dimer to monomer dissociation, and
monomer unfolding) were occurring. To assess the stability of the dimer-
dimer interface in the absence of the monomer-monomer interface, a
repressor mutant, K84L, was used. This mutant repressor protein does not
demonstrate a decrease in fluorescence or CD signal in urea concentrations
as high as 8 M (Figure 40 A & B; Nichols & Matthews, 1997). This
mutant repressor was able to bind to inducer in 8 M urea, indicating that
the monomer is still folded (Figure 40B). Since the Lys^{34} residue is located
in the monomer-monomer interface, the ability of the leucine substitution
to stabilize the protein presumably derives from stabilizing the dimer
structure (Friedman et al., 1995; Lewis et al., 1996; Chang et al., 1993;
Nichols & Matthews, 1997).
Figure 39. Operator binding curves for refolded wild type repressor. Wild type protein was renatured as described in Materials and Methods. Nitrocellulose filter binding assays were used to measure operator binding for renatured wild type protein (●), wild type protein treated in the same manner but not denatured (○), and wild type control (▲). The curves were generated using Igor Pro by fitting the data to Equation 1 in Materials and Methods.
Figure 40. Denaturation of K84L mutant repressors. A. CD signals for K84L (■) and K84L/-11 aa (□) were monitored as a function of urea. Wild type (○) repressor data for the same conditions is included for comparison. Protein concentration for both repressor proteins was 4 μM monomer. Lines are drawn through the points for comparison purposes only. B. IPTG binding activity for K84L mutant repressor in different concentrations of urea. An ammonium sulfate assay was used to determine the ability of K84L (□) and wild type (○) protein to bind IPTG in urea. The fluorescence unfolding curves for K84L (■) and wild type (○) proteins are included for comparison purposes.
Since the tetramer to dimer transition for wild type lac repressor appears to be transparent to both fluorescence and CD signals, molecular weight as a function of urea was used to monitor this transition. Under non-denaturing conditions, sedimentation equilibrium experiments using multiple speeds and concentrations demonstrated that the apparent molecular weight of K84L is 150,000 Da (Figure 41). A dimeric form of the K84L mutant, K84L/-11 aa, was used as a control for the unfolding experiments (Nichols & Matthews, 1997). This mutant should show no change in molecular weight as a function of urea if the dimeric structure remains stable throughout the concentrations of urea used in these experiments. In sedimentation equilibrium experiments using multiple speeds and concentrations, this repressor mutant fit well to an apparent molecular weight of 72,800 Da (Figure 42). This molecular weight reflects the overall loss of twenty two amino acids due to the -11 aa mutation at the C-terminus of both monomers.

At high concentrations of urea, K84L and K84L/-11 aa repressor proteins each have an observed molecular mass of ~ 65,000 Da. The K84L/-11 aa mutant protein always displayed a slightly reduced molecular weight compared to the K84L repressor because of the missing amino acids at the C-terminus. At intermediate concentrations of urea (between 4 and 5.5 M) the K84L protein displayed a decrease in apparent molecular weight. As shown in Figure 43, the fraction of dimeric protein increases
Figure 41. Sedimentation equilibrium scans for K84L mutant repressor. Two speeds (12,000 rpm and 14,000 rpm) and two concentrations (4 µM and 8 µM) were fit simultaneously to a molecular weight of 150,000 Da using a nonassociating model. The residuals for each fit versus the data points for each curve shown are plotted. The different conditions are represented by the following symbols: 4 µM at 12,000 rpm (■), 4 µM at 14,000 rpm (●), 8 µM at 12,000 rpm (□), and 8 µM at 14,000 rpm (○).
Figure 42. Sedimentation equilibrium scans for K84L/-11 aa mutant repressor. Two speeds (16,000 rpm and 18,000 rpm) and two concentrations (4 μM and 8 μM) were fit simultaneously to the molecular weight of 72,800 Da using a nonassociating model. The residuals for each fit versus the data points are shown for each curve. The different conditions are represented by the following symbols: 4 μM at 16,000 rpm (●), 4 μM at 18,000 rpm (■), 8 μM at 16,000 rpm (○), and 8 μM at 18,000 rpm (□).
Figure 43. Tetrameric to dimeric transition for K84L mutant repressor. The apparent molecular weight for K84L in different concentrations of urea was determined using sedimentation equilibrium experiments as described in Materials and Methods. The apparent molecular weight was converted into fraction of dimer, $F_d$, and these data points were fit to a two state model of $T_4 \Leftrightarrow 2D_2$ using Igor Pro as described in Materials and Methods.
with increasing urea concentration, and the transition exhibits a midpoint at
~5 M urea. Fitting these data to a two state model for a transition from
tetrameric to dimeric protein resulted in a free energy value of ~19.6
kcal/mol for dissociation into dimers. Thus, the dimer-dimer interface is
very stable when the monomer-monomer interface dissociation is
prevented by the K84L mutation.

*Analysis of tetrameric lac repressor folding*

Mutant *lac* repressor proteins have been used to determine the
stability of the two different subunit interfaces and the results of these
experiments are summarized in Table 10. The energy associated with the
monomer-monomer interface has been measured for ~11 aa dimeric
repressors as $\Delta G^\circ \approx 10.0$ kcal/mol or 14.3 kcal/mol for R3 a dimeric
repressor with the C-terminal leucine heptad repeat replaced by the
corresponding dyadic motif of GCN-4 (Chen *et al.*, 1994). A monomeric
*lac* repressor mutant, Y282D, was used to determine the contribution of the
unfolding of monomeric *lac* repressor ($\Delta G^\circ \approx 4.8$ kcal/mol) (Chen and
Matthews, 1994). The free energy of the tetramer to dimer transition
obtained from the experiments using the K84L mutant is $\Delta G^\circ \approx 19.6$
kcal/mol. The transition of tetrameric to unfolded monomeric *lac*
repressor can be described by a sequence of these transitions. The total free
energy change can be described as $\Delta G^\circ_{T-U} = \Delta G^\circ_{T-D} + 2\Delta G^\circ_{D-M} + 4\Delta G^\circ_{M-U}$
Table 10: Equilibrium Constants Derived from Denaturation Experiments

<table>
<thead>
<tr>
<th>repressor</th>
<th>$\Delta G^o$ T-U$^a$</th>
<th>$\Delta G^o$ D-U$^b$</th>
<th>$\Delta G^o$ M-U</th>
<th>$\Delta G^o$ T-D$^c$</th>
<th>$\Delta G^o$ D-M$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y282D$^e$</td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-11 aa</td>
<td>19.6 ± 0.8</td>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>m$^f$ = 4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3$^e$</td>
<td>23.9</td>
<td></td>
<td></td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>K84L</td>
<td></td>
<td>19.6 ± 0.8</td>
<td>10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>m = 2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>49.1 ± 3.3</td>
<td></td>
<td></td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m = 9.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The free energy change for tetramer to unfolded monomer was determined by fitting the fluorescence data in Figure 44 to the model described in Materials and Methods.

$^b$ The free energy change for the dimer to unfolded monomer was determined by fitting the fluorescence data in Figure 46 to the two state transition model described in Materials and Methods. These values are within error of those reported by Chen and Matthews, 1994.

$^c$ The free energy change for tetramer to dimer was determined by fitting the ultracentrifuge data in Figure 43 to the two state transition model described in Materials and Methods.

$^d$ These values were calculated by subtracting twice the contribution of Y282D from the dimer to unfolded monomer free energy change.

$^e$ The free energy values for Y282D and R3 unfolding are from Chen and Matthews, 1994.

$^f$ m value measures the dependence of $\Delta G$ on concentration.
which yields an overall calculated $\Delta G^o_{\text{T-U}} = 58.8$ kcal/mol. However, the\nlac repressor mutant proteins used to generate these values do not\nnecessarily reflect the stability of these interfaces or the folded monomer\non folding in the wild type protein.

The wild type fluorescence and CD data could be fit to a variety of\nmodels. These spectroscopic techniques only detect a single transition and\nnot the multiple equilibria between oligomeric states which might occur\nduring the unfolding of wild type lac repressor. In choosing the models\nfor fitting this data, the following two assumptions were made: 1) the loss\nof CD signal correlates with monomeric protein being completely\nunfolded; and 2) no tetramer to dimer dissociation occurs prior to the\nunfolding transition.

Two models meeting this criteria are (1) $T_4 \Leftrightarrow 4U$, and (2) $T_4 \Leftrightarrow I_4$\n$\Leftrightarrow 4U$, where $I_4$ represents unfolded monomers which are still associated\nthrough the dimer-dimer interface. Examples of simultaneous fitting of\nthe unfolding curves for wild type repressor at multiple concentrations to\nboth of these models is shown in Figure 44 A & B. Fitting the data to\nmodel 1 (Figure 44A) results in a $\Delta G^o_{\text{T-U}}$ value of 49.1 kcal/mol, but at\nhigher protein concentrations the model does not fit well to the data.

For the second model (Figure 44B), the dependence of the unfolding\ncurves upon repressor protein concentration was used to determine the
Figure 44. Wild type repressor unfolding data fit to various models. Fluorescence intensity was monitored as a function of urea at four different monomeric concentrations of wild type repressor, 4 μM (Δ), 2 μM (▲), 1 μM (○), and 0.5 μM (◆). The curves were generated by simultaneously fitting all of these data sets. A. Unfolding curves generated by fitting the data to a $T_4 \Leftrightarrow 4U$ model. B. Unfolding curves generated by fitting to the data to $T_4 \Leftrightarrow I_4 \Leftrightarrow 4U$ model.
contribution of each step to the total free energy of the transition. In this model, the first transition \((T_4 \Leftrightarrow I_4)\) would be insensitive to concentration, while the second transition \((I_4 \Leftrightarrow 4U)\) would display concentration dependence. Therefore, the contribution of each step to the overall unfolding free energy could be determined by fitting unfolding curves at different concentrations simultaneously. These fits result in a free energy for the \(T_4 \Leftrightarrow I_4\) transition of 13.5 kcal/mol and a free energy of 35.6 kcal/mol for the \(I_4 \Leftrightarrow 4U\) transition.

It is possible that the complete dissociation of \(I_4 \Leftrightarrow 4U\) occurs past the transition monitored by fluorescence and CD. If the fluorescence and CD transitions do not monitor the complete unfolding of the monomer, it would account for both the lower free energy value determined for the total free energy of wild type unfolding and the reduced concentration dependence detected for this transition. Figure 45 demonstrates the two different pathways to calculate the free energy for the tetrameric lac repressor to unfolded monomer transition.

*Ligand binding alters the stability of lac repressor subunit interfaces.*

The presence of IPTG has previously been shown to stabilize *lac* repressor structure (Schnarr & Maurizot 1981, Royer et al., 1990a), while operator binding may destabilize the tetrameric structure but stabilize the dimeric form of the protein (Royer et al., 1990a). The effect of operator
Figure 45. Wild type repressor folding pathways. Two different pathways by which wild type repressor can assemble into a tetrameric structure are shown. Data were collected corresponding to different steps in these pathways. However, the overall ΔG°_{T-U} for each pathway does not match for these two routes.
binding on stability could not be assessed in these experiments because repressor proteins do not bind operator in the presence of urea. The K84L protein which should maintain its dimeric structure in high concentrations of urea did not bind operator at 1 M urea (data not shown). Whether this is a result of helix-turn-helix unfolding or the urea interfering with DNA binding is undetermined.

Ammonium sulfate assays demonstrate that lac repressor dimer can bind IPTG in urea (Chen & Matthews, 1994). The presence of IPTG shifts the transition region of both -11 aa and wild type repressor unfolding curves towards higher concentrations of urea which indicates that both are stabilized by binding IPTG. In the presence of IPTG, -11 aa repressor protein had a $\Delta G^\circ_{D,U} = 23.8$ kcal/mol (Figure 46), and wild type repressor has a $\Delta G^\circ_{T,U} = 60.3$ kcal/mol (Figure 47). Therefore, the binding of IPTG stabilized the -11 aa repressor protein by 4 kcal/mol compared to the free -11 aa repressor protein, and wild type protein was stabilized by 11 kcal/mol compared to the unliganded wild type repressor.

Discussion

Lac repressor binds to operator DNA and prevents transcription of the genes necessary for lactose metabolism. One aspect of this repression is the ability of tetrameric lac repressor to bind DNA at two sites and
Figure 46. Unfolding of −11 aa mutant repressor in the presence of IPTG. Urea denaturation was monitored by fluorescence in the presence of 1 mM IPTG at one protein concentration, 4 μM (○) monomer. The data were fit in Igor Pro to the $D \Leftrightarrow 2U$ model described in equation 9 in Materials and Methods. The −11 aa unfolding curve fit at 4 μM (●) monomer based on Figure 48 is included for comparison.
Figure 47. Unfolding of wild type repressor in the presence of IPTG. Urea denaturation was monitored by fluorescence in the presence of 1 mM IPTG at two protein concentrations, 1 μM (○) and 4 μM (△) monomer. The data were fit simultaneously to the $T_4 \Leftrightarrow 4U$ model described in Materials and Methods. The solid line represents the fit for the wild type repressor unfolding curve at 1 μM (●) monomer protein concentration from Figure 44.
produce loops (Eismann & Müller-Hill, 1990; Oehler et al., 1990). To form loops, two different interactions must occur. The monomeric protein must be assembled into dimers which interact with single operator sites and the dimeric protein must assemble into tetramers which allow two operator sites to be occupied simultaneously by one protein molecule. These processes therefore incorporate both protein-protein as well as protein-DNA interactions.

Urea denaturation was used previously to determine the overall stability of lac repressor subunit interface mutants (Chen and Matthews, 1994). These experiments demonstrate that urea denaturation for -11 aa and R3 mutant proteins occurs via a dimer to unfolded monomer transition. Evidence for this concerted transition is given by the simultaneous loss of CD and fluorescence signals, and IPTG binding activity. The concentration dependence of the unfolding curves also indicates that an assembly process is being monitored. Figure 48 shows examples of the concentration dependence for unfolding -11 aa by urea. Fitting these curves simultaneously to a $D_2 \leftrightarrow 2U$ transition resulted in a free energy value of 19.6 kcal/mol which is within experimental error of the previously reported value of 19.3 kcal/mol (Chen & Matthews, 1994).

A second lac repressor mutant was used to determine the free energy involved in the dimer-dimer interface. Mutation of Lys$^{84}$ to alanine or leucine resulted in a repressor protein which maintained its dimer structure
Figure 48. Concentration dependence of -11 aa mutant repressor unfolding. Fluorescence data were collected for unfolding experiments at four different concentrations of mutant repressor, 4 μM (▲), 3.5μM (Δ), 1 μM (○), and 0.25 μM (●) monomer. The curves were generated by simultaneously fitting all of these data sets to a two state $D_2 \rightleftharpoons 2U$ model in Igor Pro as described in Materials and Methods.
in 8 M urea (Nichols & Matthews, 1997). Therefore, in the absence of the dimer to monomer transition, the free energy associated with the tetramer to dimer transition could be measured. Sedimentation equilibrium experiments monitored molecular weight as a function of urea, and these data were fit to a model of $T_4 \Leftrightarrow 2D_2$. Combining the -11 aa mutant data for the $D_2 \Leftrightarrow 2U$ transition and the K84L mutant data for the $T_4 \Leftrightarrow 2D_2$ transition results in a $\Delta G^*_{T-U} = 58.8$ kcal/mol for the transition of lac repressor tetramer to unfolded monomer.

Only a single transition is detected using both fluorescence and CD measurements for wild type unfolding. Because no dissociation to dimer at low concentrations of urea was detected in sedimentation equilibrium experiments, the simplest model which fits this data is $T_4 \Leftrightarrow 4U$. However, there are indications that this model may not represent all the processes which are occurring during unfolding. A fourth order dissociation event should result in greater concentration dependence than seen for the wild type unfolding curves. The total free energy calculated for the repressor to unfolded monomer from these data is less than that calculated by adding all of the values for the subunit interface mutant repressors. These inconsistencies indicate that a more complex model may be necessary to describe the fluorescence and CD transitions.
The second model to fit the fluorescence and CD data incorporated the formation of an intermediate, I₄. Based upon the free energy values determined for the subunit interface repressor mutants, the dimer-dimer interface could persist after the monomer-monomer interface has dissociated. Therefore, this intermediate consists of the unfolded monomers still associated at the dimer-dimer interface. The free energy value determined by fitting the data to this model still results in a lower than expected overall total free energy compared to adding the free energies for the subunit interface mutants. However, this lower value could be a result of the fluorescence and CD data not monitoring the complete I₄ ⇌ 4U transition. Since the completely unfolded monomer is not detected in this experiment, the free energy determined from fitting these data is less than expected.

The presence of this tetrameric intermediate at 2.5 to 3 M urea could not be detected in sedimentation equilibrium experiments. After the pretransition baseline, no consistent data could be collected for the higher concentrations of urea. This situation is also true for -11 aa and indicates that the problem is not unique to the presence of the dimer-dimer interface. The extended period of time required for samples to reach equilibrium in the ultracentrifuge may allow aggregation of a folding intermediate. If even a small amount of partially folded protein is susceptible to aggregation, an alternative pathway to refolding will occur which is
essentially irreversible. It is possible that the presence of IPTG increases renaturation yields by stabilizing the monomer fold and reducing the occurrence of these intermediates. As long as these pathways do not predominate in the time frame for unfolding experiments, equilibrium information can be obtained in the fluorescence and CD experiments.

A gradual decrease in the CD signal between 4 and 6 M urea was detected for both -11 aa and wild type repressor protein. Previous experiments which measured the stability of lac repressor using urea denaturation also observed a gradual transition of the CD signal. Schnarr and Maurizot (1981) used urea denaturation experiments to determine the relative stability of wild type lac repressor, the core protein, and the N-terminal helix-turn-helix domain. Both the core and wild type proteins demonstrated the gradual decrease in signal from 4 to 6 M urea. However, this transition was not as steep for the core as for the wild type repressor. Since the N-terminal fragment displayed CD signal up to 8 M urea, Schnarr and Maurizot concluded that this second transition represented the N-terminus unfolding. Even though this transition is in the region where K84L dissociates into dimers, the data presumably contains no information about the dimer-dimer transition since this gradual decrease is also seen for -11 aa.

Lac repressor stability has also been investigated using pressure to dissociate the protein while monitoring the unfolding transition by
fluorescence polarization (Royer et al., 1986; Royer et al., 1990a). These experiments used wild type repressor and a dansyl-\textit{lac} repressor for the lower concentration studies. Unfolding of wild type and modified repressor resulted in an estimated equilibrium dissociation constant for the wild type tetramer to dimer transition of $1 \times 10^{-8}$ M to $1 \times 10^{-9}$ M. These values result in a much lower free energy estimate than values obtained from the urea denaturation experiments presented in this thesis and are contradicted by operator binding experiments (see below; Brenowitz et al., 1991b; Levandoski et al., 1996). In the pressure denaturation experiments, assumptions inherent in extrapolating the data back to atmospheric pressure, the oligomeric state of the transition end point, and the reversibility of the reaction, all could contribute to the lower apparent free energy values.

Operator binding studies provide the most conclusive evidence that \textit{lac} repressor is a tightly assembled tetramer. The multiple methods of detecting binding events, such as gel shift and nitrocellulose filter binding, allow for detection of different binding species. The sensitivity of these assays permit low concentrations of protein to be assessed. Only tetrameric \textit{lac} repressor can form DNA loops, and this behavior has been monitored to provide an estimate for the strength of the dimer-dimer interface. Brenowitz et al. (1991b) used footprint titration analysis and gel shifts to correlate double site occupancy with looped or tandem DNA-protein
complexes. These data were fit to the tetramer to dimer dissociation constant reported by Royer et al. (1990a) of $1 \times 10^{-8}$ M, an intermediate value of $1 \times 10^{-10}$ M, and a value where essentially no dissociation was occurring of $1 \times 10^{-19}$ M. The binding data did not fit well to the model when the two extreme values for tetramer to dimer transition were used; however, the data could be fit to any value between $1 \times 10^{-10}$ to $1 \times 10^{-18}$ M. These experiments provide a range for the anticipated dissociation constant for tetrameric repressor in looped complexes.

The presence of DNA loops can be directly monitored by tracking the Brownian motion of beads attached to DNA fragments. The formation of loops removes the beads from detection which allows the rate of looping and unlooping to be measured directly. The Gelles laboratory monitored the formation of loops by lac repressor for DNA fragments with two operator binding sites. Their conclusion was that a tetramer to dimer equilibrium did not contribute to the unlooping of the DNA (Finzi & Gelles, 1994). This conclusion was based upon the fact that the measured rates for unlooping correlated with the off rates for lac repressor binding to operator under similar conditions (Hsieh et al., 1987). If a tetramer to dimer dissociation does not affect looping, these protein-protein interactions must be stronger than the operator-protein interactions.

Gel shift assays analyzing the tetrameric lac repressor bound to fragments of DNA with single operator sites were also used to detect the
presence of lac repressor tetramers. These experiments eliminated any
effects that looping might have on stabilizing the tetrameric structure.
Fickert & Muller-Hill (1992) determined the fraction of tetrameric wild
type protein present in solution by detecting the presence of complexes
which were associated to two operator fragments at saturating
concentrations of DNA. They found that at $3 \times 10^{-11}$ M, more than 80% of
the complexes were tetrameric, while at $3 \times 10^{-12}$ M only 30-50% were still
tetrameric. These estimates are lower than the values currently accepted
for the stability of the tetramer. These experiments may have detected
lower amounts of tetramers because of the small concentration range over
which binding was monitored.

The Record laboratory has extensively modeled filter binding
experiments using different concentrations of ligand and protein to detect
the contribution of dimeric lac repressor protein binding to operator DNA.
A thermodynamic model incorporating the presence of dimer-operator,
tetramer-operator, and tetramer-2 operator species for fitting binding
isotherms did not detect the presence of dimer-operator binding even in the
femtomolar protein concentration range. These experiments set a
concentration limit for lac repressor tetramer dissociation and indicate that
lac repressor is tetrameric in the concentration range in which it interacts
with DNA. Our estimate of $\sim 10^{-15}$ M for the tetramer to dimer transition
is consistent with these results.
The presence of ligands can stabilize protein structures against chemical denaturants. While the effect on operator cannot be determined in these assays, IPTG binds to lac repressor in urea and alters the midpoint of the urea-induced unfolding transition. Previously, Schnarr and Maurizot (1981) reported that both core and wild type repressor were stabilized by IPTG. Optimization of wild type refolding conditions allowed the free energy change to be calculated for both wild type and -11 aa. In these experiments, the magnitude of the change in stability, 11.0 and 4.2 kcal/mol for wild type and -11 aa respectively, is similar to that found for other proteins. Binding of tryptophan to trp repressor resulted in a free energy change in the stability of the protein of 6 kcal/mol, and binding of a peptide inhibitor resulted in HIV-1 protease being stabilized by 5 kcal/mol (Fernando and Royer, 1992; Grant et al., 1992). It is unknown for lac repressor whether IPTG binding stabilizes contacts in the monomer-monomer interface or the monomeric protein.

The pressure dissociation experiments were able to determine the effect of operator and inducer on the stability of lac repressor and the danysl-lac repressor. Royer et al. (1990a) reported that the presence of IPTG increases the stability of wild type repressor by ~0.8 kcals. This value is lower than the changes detected in urea denaturation assays. Both high pH and bound operator destabilized tetramer, and a transition to a
stable dimer was deduced. These results are consistent with high pH and operator binding having the same effect on inducer binding.

The lac repressor subunit interface mutants allow the contribution of each interface to be analyzed and permit speculation about how the lac repressor assembles both in vitro and in vivo. Figure 45 demonstrates the different energies determined for each step of the pathway for lac repressor folding and assembly. In this scheme, the dimeric structure is never present because its free energy is lower than that for tetramer association. These values correlate with functional experiments which indicate that lac repressor does not exist as a dimer even at low concentrations in vitro. For lac repressor, if the dimer-dimer interface assembles before the monomer folds and assembles into dimers, the monomers are restricted to a specific partner monomer. In the dimer mutants, the monomer has the ability to fold with any partner. This order of association maybe the reason wild type folding must occur at low concentrations.

These unfolding studies demonstrate the difficulties of refolding proteins in vitro which possess two interfaces with different stabilities. In vivo, the N- to C-terminal synthesis of proteins may prevent this assembly problem. Attachment to the ribosome may allow the dimer to form before it associates into it a tetramer. Therefore in the cell, this reaction would be second order and less complicated than the fourth order reaction which
occurs \textit{in vitro}. The overall stability and strength of the tetramer interface indicates that \textit{lac} repressor does not exist as a dimer in the cell and that the tetramer to dimer equilibrium does not play a regulatory role in looping.

In conclusion, the \textit{lac} repressor protein is an extremely stable tetramer which does not dissociate at protein concentrations in which it interacts with operator DNA fragments. The two approaches used to determine the overall stability of the tetrameric repressor indicate that different methods used to monitor the urea denaturation of the protein detect different unfolding transitions. The free energy of complete unfolding by urea-induced denaturation monitored by CD and fluorescence for the tetrameric repressor is \(\sim 49.1\) kcal/mol. However, the total free energy for denaturation of tetrameric repressor calculated by combining all of the assembly and folding events is \(\sim 58.8\) kcal/mol. The data from the two different approaches allows for speculation about how the different subunit interfaces may contribute to the overall stability of the \textit{lac} repressor protein.
Appendix

Monod-Wyman-Changeux Model

The Monod-Wyman-Changeux model allows the ligand binding behavior of a protein to be expressed in terms of structural changes. This model has been used to explain how the binding of one ligand by lac repressor alters the affinity for the second ligand. The ability of the protein to have a different affinity for the same ligand is based upon the protein existing in two different structural conformations: an R state which has a high affinity for inducer and a low affinity for operator, and a T state which has a high affinity for operator and a low affinity for inducer. In the absence of ligands, the protein exists in an equilibrium between both conformations. The relative concentrations of each state when no ligand is present is described by an equilibrium constant, L. A large L value indicates that the unliganded protein prefers the T state while a small L value indicates that the protein prefers the R state. An L value of unity indicates that the unliganded protein exists equally in both conformations.

The model which describes this behavior requires the following assumptions: 1) the repressor protein possess at least one axis of symmetry; 2) each of the ligands (i.e., inducer, operator DNA fragment) binds to only
one site on the repressor monomer; 3) the conformation of each monomer is limited by its interaction with the other monomers; 4) two reversibly attained states of the repressor tetramer are available (R and T states) which have different affinities for inducer and operator DNA fragments, and; 5) the symmetry of the protein is conserved in the transition from one state to the other. For the lac repressor protein, the transition states available to the repressor are represented in Scheme 1. This scheme represents the repressor protein binding to two operator DNA fragments.

To predict the fractional saturation of the repressor protein at varying inducer concentrations for a tetrameric protein which binds to four inducer molecules and two operator DNA fragments, the following equation can be used:

\[ Y = \frac{\left[ K_{RI}[I](1 + K_{RO}[O] + K_{TR}^2[O]^2) + LcK_{RI}[I](1 + cK_{RI}[I])^2(1 + 2K_{TO}[O] + K_{TO}^2[O]^2) \right]}{\left[ (1 + K_{RI}[I])^4(1 + 2K_{RO}[O] + K_{RO}^2[O]^2) + L(1 + cK_{RI}[I])^4(1 + 2K_{TO}[O] + K_{TO}^2[O]^2) \right]} \]

In this equation, \( Y \) is the fraction of inducer-bound complexes at a specific concentration of inducer, \( K_{RI} \) is the association constant for the binding of inducer to the R state, and \( c \) is the ratio between the association constants for the T and R states for inducer \( (K_{RT}/K_{RI}) \) and reflects the difference in inducer affinity for these two states. \( K_{RO} \) is the association constant for operator DNA binding to the R state and \( K_{TO} \) is the association constant for the T
Scheme 1. Monod-Wyman-Changeux model for lac repressor binding of inducer and operator DNA. This scheme incorporates two operator binding sites and demonstrates all possible liganded states of the lac repressor protein. I and O refer to inducer and operator, and the R and T states are described in the text.
state. The equilibrium constant, $L$, represents the relative concentrations of the R and T states when no ligand is present ($L = T/R$). The model uses intrinsic binding constants for these equilibrium association values. The intrinsic binding constants represent the affinity of the protein for the ligand in the absence of allosteric effects. The values used to fit the data were $K_{RI} = 7.1 \times 10^5 \text{ M}^{-1}$ (inducer affinity in low pH), $K_{II} = 7.7 \times 10^3 \text{ M}^{-1}$ (inducer affinity in the presence of operator at high pH), $K_{RO} = 6.3 \times 10^6 \text{ M}^{-1}$ (operator affinity in the presence of inducer at low pH), $K_{TO} = 1.7 \times 10^9 \text{ M}^{-1}$ (operator affinity in high pH) and $c = 0.011$ (Daly & Matthews, 1986a). The $L$ value was allowed to float to determine the relative concentration of the R and T states.
References


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