NMR investigation of non-local effects in a temperature sensitive mutant of the 25 kD tryptophan repressor protein
by Robert Charles Tyler

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Science
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Robert Charles Tyler

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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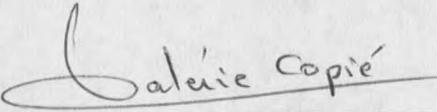
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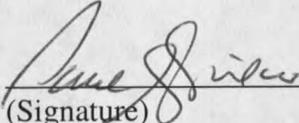
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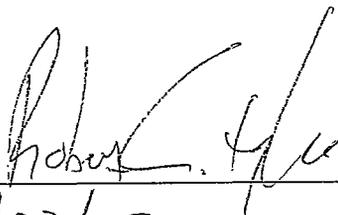(Signature)   Date

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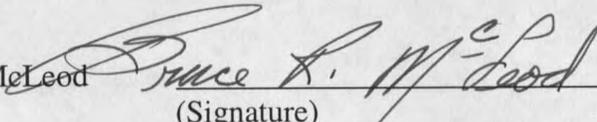
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Chapter 1

INTRODUCTION

The tryptophan repressor protein (TrpR) of *Escherichia coli* is a DNA binding protein that regulates transcription of genes that control L-tryptophan (L-trp) biosynthesis. The activity of TrpR is modulated by the intracellular concentration of its cofactor L-trp. When the cell contains high levels of L-trp, the inactive, unliganded form of the protein (apo-TrpR) binds two molecules of L-trp, which results in the active form (holo-TrpR). This activated holo-form of the protein then binds to specific operator DNA sequences pertinent to the biosynthesis of L-Trp, thereby preventing transcription. The TrpR is a 25kDa homodimer with each subunit containing 108-residue polypeptide chain (*1*). Structural studies have shown (*2-6*) that each subunit consists of six α-helices labeled as helices A-F. The TrpR is comprised of two structural domains, a hydrophobic core formed by helices A, B, C, and F of both subunits, and a DNA binding domain formed by helices D and E. The two molecules of L-trp bind to the dimer at sites formed between the hydrophobic core and the DNA binding domain.

From the first structural study of TrpR carried out by Schevitz et al (*2*), and later crystallographic comparisons of apo/holo forms (*3*), it was generally thought that binding L-Trp cofactor properly orients helices D and E away from the hydrophobic core for specific interactions within successive major grooves of target DNA. Indeed,

the holo/DNA crystal structure (*7*) confirms that the DNA binding domain of TrpR inserts into successive major grooves of operator DNA. Nuclear magnetic resonance (NMR) solution studies of the different forms of TrpR (*4-6*) have revealed more pronounced effects on the TrpR structure within helices D and E upon binding L-trp and DNA.

Protein structures based on solution NMR techniques rely heavily on the nuclear Overhauser effect (nOe) observed between protons. The nOe is a consequence of the dipolar interaction between nuclei, and is proportional to the inverse sixth power of the distance separating the nuclei. The nOe represents a through-space magnetization transfer from one nuclei to another. More importantly the nOes are sensitive to molecular conformation, and measurement of the nOe allows one to estimate the distance between two nuclei. The nOe, as it pertains to protein NMR, is measured as the cross-peak intensity between two interacting protons, and the greater the intensity of the nOe cross-peak, the shorter the distance separating the two nuclei. The nOe between two nuclei has an interaction range of about 5.5 Å. Once the distance between the two nuclei is estimated, it can be used to define a structural restraint within the protein. Hundreds of nOe structural restraints, along with many other NMR restraint parameters that describe backbone dihedral angles, and hydrogen bonding patterns, are then used to calculate the three dimensional structure of the protein and permits analyses by NMR spectroscopy.

From solution NMR structural investigations, both apo- and holo-TrpR have similar tertiary folds to the ones found in the corresponding crystal structures (2-6).
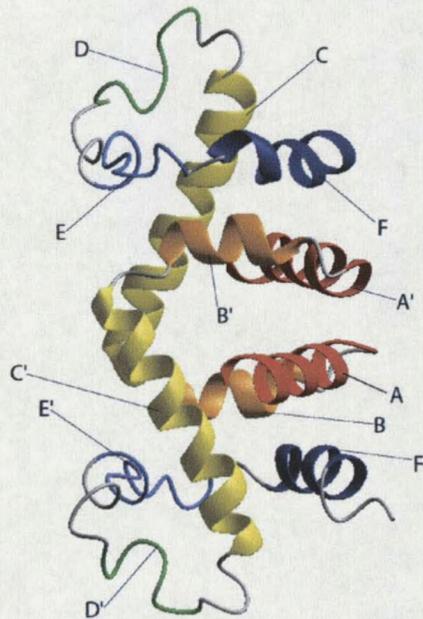
Figure 1: Minimized average structure of apo-WT TrpR based on the work of Zhao et al (5). Helical regions are represented as ribbons with the following boundaries: helix A/A' residues 16-32, helix B/B' residues 37-42, helix C/C' residues 45-63, helix D/D' residues 68-74, helix E residues 81-90, and helix F residues 93-103.

However, analysis of NMR solution structures reveals that the DNA binding domain, i.e. helices D and E, is more flexible and disordered than in the crystal structure (4;5). In particular, Zhao et al (5) have shown that conformational determinations of helices D and E are difficult to perform for apo-TrpR due to a lack of nOe structural restraints. Figure 1 shows a representation of wild-type apo-TrpR based on the NMR structures of Zhao et al (5). The binding of the corepressor L-trp to the protein produces more pronounced nuclear Overhauser effects (nOe) which are characteristic of a better

defined helix E in holo-TrpR. Similarly, more intrahelical nOes are observed in helix D in the holo-TrpR/DNA ternary complex than in DNA-free holo-TrpR and indicate that when holo-TrpR binds to the DNA operator sequence, helix D becomes better defined in solution (*6*).

The lack of order in the DNA-binding region of TrpR, reflected in the paucity of nOes observed in this area, can be further characterized by considering the dynamics of the protein's architecture. Comparison of backbone amide exchange rates revealed pronounced differences between the DNA-binding domain and the hydrophobic core of the protein (*8-11*). The exchange rates observed within the DNA-binding region were orders of magnitude faster then amide groups located within the hydrophobic core. Binding of L-trp caused a slowing of exchange rates in all regions of the protein. Backbone amide groups that exchanged rapidly in apo-TrpR also exchanged fast in holo-TrpR, however the rates were approximately 3 times slower for holo-TrpR (*9*). A more dramatic effect of L-trp binding was observed for the slower exchanging amide groups of the hydrophobic core. The exchange rates observed in this area of holo-TrpR are an order of magnitude slower than corresponding amide groups in apo-TrpR. These results demonstrated that regional differences in dynamics exist within both forms of TrpR, and that binding of L-trp produces global dynamic effects throughout the protein. These experiments provided direct evidence that the DNA-binding domain of TrpR is more flexible, which had been suggested by NMR structural studies (*4;5*).

The backbone dynamics of TrpR were also probed by ^{15}N NMR relaxation analysis by Jardetzky and coworkers (*12*). In this study the relaxation properties (T_1, T_2, and

steady state nOes) of ^{15}N nuclei found within the TrpR backbone were measured. The methodology and theory associated with these types of measurements will be presented in greater detail in Chapter 2 (Materials and Methods). Equations that described NH backbone movement were fitted to ^{15}N relaxation data and provided insight into the motional restrictedness of individual NH bond vectors. This type of analysis allowed a detailed description of NH bond vectors in terms of an amplitude and timescale of motion. To facilitate reliable descriptions of these motional parameters, researchers had to find an accurate model of the rotational diffusion that represents the TrpR protein. This is due to the fact that the theoretical assumptions which allow for the motional characterization of the NH bond vectors within the backbone are dependent upon rotational diffusion of the protein (*13;14*). Rotational diffusion represents the frequency of protein reorientation in solution, which is dependent upon its shape. Conventionally rotational diffusion is described by a 3x3 matrix called a tensor, which when diagonalized produces the principal values of the diffusion coefficients, often written as D_{xx}, D_{yy}, and D_{zz}. These coefficients represent the magnitude of rotation about their respective axis. ^{15}N NMR solution studies approximate these coefficients, the diffusion tensor, by fitting possible diffusion models to experimental ^{15}N T_1/T_2 ratios (*15*). Jardetzky and coworkers found that the rotational diffusion tensor of TrpR was best modeled as an anisotropic axially symmetric prolate ellipsoid (*12*). In this case, the value of D_{xx} is greater than D_{yy} or D_{zz}, and the value of D_{yy} is equal to D_{zz}. For this type of diffusion tensor D_{xx} is referred to as $D_\parallel$, with D_{yy} and D_{zz} referred to as $D\perp$. The ^{15}N NMR relaxation data collected on holo-TrpR by Zheng et al were consistent with $D_\parallel$

and $D\perp$ values of 1.48 ± 0.06 s^{-1} and 1.15 ± 0.05 s^{-1}, respectively. Prior to this work (1995) many ^{15}N NMR relaxation studies (16-18) simply assumed isotropic rotational diffusion, which sets D_{xx}, D_{yy}, and D_{zz} all equal. This assumption models the proteins shape as a sphere. However, this can led to significant errors in dynamic analysis when deviations from spherical shape influence the measured relaxation properties of the protein (19). This deviation is measured by the ratio $D_\parallel/D\perp$ and is referred to as the anisotropy of the protein. When the protein shape is modeled as a sphere (i.e isotropic rotational diffusion) the ratio $D_\parallel/D\perp$ is equal to one. Any $D_\parallel/D\perp$ ratio greater than one represents anisotropic axially symmetric rotational diffusion and alters the theoretical equations one must use to probe the internal backbone motions. The $D_\parallel/D\perp$ ratio calculated for holo-TrpR was 1.28 and clearly demonstrated that anisotropy should be considered when probing internal backbone motions. Once the diffusion tensor was correctly established, the amplitude and timescale for the internal motion of individual NH bond vectors within TrpR were determined. The work of Jardetzky and coworkers showed that the entire Trp Backbone, excluding the N and C termini, is quite rigid on the nanosecond time scale (12). Unlike the amide exchange results, the ^{15}N dynamic study indicated that the DNA-binding domain is as rigid as the hydrophobic core on the nanosecond timescale. This finding demonstrated that there is no independent motion of backbone NH bond vectors within the DNA-binding domain relative to the hydrophobic core, suggesting that as the protein tumbles in solution these two regions move as a unit. The only areas of holo-TrpR that were found to contain large amplitude motions, indicative of flexibility, were the N and C termini.

The contrast between the amide exchange results and the ^{15}N relaxation study made it necessary to distinguishing between "true" flexibility and "apparent" flexibility observed within the TrpR structural domains (12). From this perspective, true flexibility is thought to be reflected in both rapid amide exchange and large amplitude motions of NH bond vectors. While on the other hand apparent flexibility was thought to be reflected in the observation of rapid amide exchange rates, but not observed as large amplitude motions of NH bond vectors in ^{15}N relaxation analysis. Taking these two definitions into account it was evident that the terminal domains of holo-TrpR represented true flexibility of the protein structure, while the DNA-binding domain demonstrated only apparent flexibility. It was speculated by Jardetzky and coworkers (12) that the apparent flexibility of the DNA binding domain represents a structural instability which manifests only on the longer millisecond timescale of amide exchange experiments. The importance of these findings is that they showed differences in backbone flexibility exist within the TrpR protein. The terminal domains reveal significant flexibility, with the hydrophobic core displaying rigid structural elements. The results of amide exchange and ^{15}N relaxation experiments suggest that the flexibility of the DNA binding domain is in between these two extremes, with amide exchange experiments pointing toward flexibility on the millisecond timescale, and with ^{15}N relaxation experiments indicating that the region is rigid on the nanosecond timescale. This characteristic difference in flexibility of the DNA binding domain could help explain the ill-defined nature of this region of TrpR that had been observed in NMR structural studies.

As mentioned previously, the results from NMR structural calculations revealed the DNA binding domain of apo-TrpR appears disordered, and that the binding of L-trp and DNA produces more nOes consistent with the presence of helical secondary structure. However disorder in this case reflects a lack of nOe structural restraints. The absences of nOes may be due to a high degree of flexibility or due to fast chemical exchange that would prevent the nOes in that region from being observed. Flexibility in this instance should manifest in both rapid amide exchange and in ^{15}N relaxation studies as large amplitude motions. It has been shown that this definition of true flexibility does not apply to the DNA binding domain. Chemical exchange is a process where a nucleus experiences different electronic environments and results in two magnetically distinct sites with different resonance frequencies. The results of a chemical exchange process can led to a broadening of NMR line shape (20) and reduce observable nOe interactions between nuclei. The amide exchange rates observed in the DNA binding domain of TrpR supported the notion that chemical exchange processes involving the amide protons are likely the cause of reduced nOes observed in this area (12). This does not mean that this area lacks alpha helical structure, but only that observation of nOes is obscured by exchange processes. In fact, chemical shift indexing (CSI) of the alpha protons of TrpR have shown some helical character within the DNA binding region (5). The chemical shift of a nucleus is a measure of the unique resonance frequency that it experiences and is sensitive to spatial conformation. When a polypeptide contains helical structure, the alpha protons contained within experience a common change in their chemical shift relative to a random coil value due to systematic

spatial conformations within the helical structure. It is possible to infer elements of helical secondary structure from NMR spectroscopy based on the indexing of these chemical shift differences (*21*).

These findings have led to the view that the DNA binding region in solution is best represented as helical structures (helix D and E) that undergo partial opening (*10*). This partial opening does not represent complete unraveling of the helices, but rather an opening of hydrogen bonds without a departure from an overall helical conformation which is evident from CSI results (*5*). This description best reconciles the findings of both amide exchange and ^{15}N relaxation results observed in the DNA binding domain of TrpR. As this region undergoes partial opening, amide protons are unprotected against rapid solvent exchange, while at the same time an overall helical conformation preserves alpha proton CSI results. This point stresses the importance of time scale as it relates to structure and stability of this region. It should be noted that stability in this case reflects the observation of properties consistent with structural elements, such as protected amide exchange, nOes, and CSI results, which suggest an alpha helical conformation. When considering this region on a millisecond time scale (*9;10*) which is reflected in the amide exchange rates, helices D and E are unstable, but not generally unstable on a nanosecond time scale reflected in ^{15}N relaxation measurements (*12*).

The structural and dynamic investigations of TrpR have led to many important factors that govern the protein's ability to function. Early crystallographic studies of TrpR defined the relationship between structural features of the protein and its cofactor/DNA binding properties. NMR solution studies of TrpR have demonstrated

that dynamics play an equally important role in the ligand and DNA binding processes. Specifically they have shown that a reduction of dynamics occurs upon ligand binding (*8;9*), and results in a stabilization (previously defined) of helix E. A calorimetric binding study of TrpR to its cofactor is consistent with NMR structural data (*22*), and also suggests that conformational change accompanies helix E upon ligand binding. Similar investigations of the holo-TrpR-DNA solution complex have shown a stabilization (previously defined) of helix D is associated with the DNA binding event (*6*).

Significant insights into the workings of TrpR have also been attained via mutational studies. Early on, mutational research was pivotal in supporting the helix-turn-helix model of repressor/DNA recognition (*23*). Through these efforts, it was also discovered that several of the isolated mutants, classified as super-repressors, were able to function at lower intracellular tryptophan concentrations (*23*) relative to wild-type repressor. Four of these super-repressors were the result of a charge change mutation and are denoted here as E13K, E18K, E49K, and D46N. (The convention used is single letter amino acid abbreviation of wild-type protein, followed by codon number, followed by the mutant residue). A fifth super-repressor resulted from a neutral charge change and is represented as A77V. It is interesting to note that each point mutation is located in one of the distinct structural domains of the protein, yet all share a super repressor phenotype. For instance, E13K resides in the unstructured N-terminal arm, while E18K, E49K, and D46N are found in the hydrophobic core of the protein. A77V, on the other hand, is located in the DNA binding domain between helix D and helix E.

In some way each of these mutants is more sensitive to intracellular tryptophan levels *in vivo*, yet a cogent understanding of the relationship between mutation and activity remained illusive.

The inspired work of Barry Hurlburt and Charles Yanofsky (*24*) investigated the relationship between mutation and activity by searching for measurable differences within these five mutant proteins. Specifically, they were looking at *in vitro* variations of ligand and operator binding as a possible explanation for super-repressor activity. Their studies revealed that the charge change mutants bound a synthetic 43 base pair operator-containing DNA fragment with a lower equilibrium dissociation constant (K_d) of $\sim 10^{-11}$ M, relative to a wild-type value of $\sim 10^{-10}$ M. Also, the concentration of L-trp needed to activate the charge change mutants (K_{act}) is well below the 10μM threshold required for wild-type repressor. Paradoxically they found that A77V produces identical values of K_d and K_{act} when measurements are compared to wild-type TrpR values *in vitro*, but displays super-repressor phenotype *in vivo*. The work of Hurlburt and Yanofsky concluded that rapid dissociation of repressor from operator targets is critical for TrpR function *in vivo*, and that favorable electrostatic interaction of the charge change mutants decreases this dissociation, which resulted in increased operator affinity (*24*).

Extensive physical characterizations of the A77V mutant were carried out by Ross Reedstrom and Catherine Royer (*25*), and identified many distinct aspects of the mutant protein. Their investigations revealed that A77V exhibits a 10% increase in apparent α-helicity as measured by CD, and 2.1 kcal/mol more stable to chemical denaturation by

urea. This CD data as well as calorimetric data strongly indicated that apo-A77V is more folded then apo-TrpR, and that binding L-trp is no longer coupled to the ordering of the DNA binding domain. It should be stated that calorimetric binding study of apo-A77V to L-trp revealed an identical affinity for cofactor binding when compared to apo-TrpR. While the cofactor binding affinities are identical (measured as Gibbs free energy, ΔG), the contributions from enthalpy (ΔH) and entropy (ΔS) are distinct for A77V. The A77V shows a much reduced unfavorable entropic contribution to ligand binding when compared to TrpR. This reduction in unfavorable entropic contribution, along with other spectroscopic data (25), suggested that the DNA binding domain of apo-A77V exists in a pre-folded state that is consistent with holo-TrpR. It was previously shown (26) that apo-TrpR dimers will self aggregate into higher order multimers at micro-molar concentrations, and that binding L-trp greatly reduces this self association process. By linking a dansyl label to the N-terminal end of the mutant protein, Reedstrom and Royer demonstrated that apo-A77V had a much lower propensity to form these higher order aggregates, and the addition of L-trp had no effect on protein self association. Once researchers considered all the unique characteristics of the mutant protein, they began to speculate about how conformational differences within apo-A77V could result in a super repressor phenotype. It was clear that the binding of L-trp produced a stabilization (previously defined) within the DNA-binding domain of TrpR, and that the A77V mutation seemed to uncouple these events. They reasoned that since ligand binding affinities of A77V and TrpR are identical due to compensation of enthalpy/entropy terms, this binding event, while important, is not

coupled directly to protein function through ligand binding affinity (*25*). This reasoning led Royer and coworkers to consider additional interactions connecting ligand binding events to operator binding, since the A77V mutation affects function. The measurements of protein aggregation provided the insight into such an interaction. Their hypothesis was that cells expressing apo-A77V would contain lesser amounts of higher order multimer protein as compared to a cells containing wild type apo-TrpR. This would result in lowering the concentration of L-tryptophan required to produce effective levels of holo-A77V capable of binding operator targets.

Numerous studies have revealed many factors that are critical for TrpR function, which include: protein-protein, protein-ligand, and protein-DNA interactions (*22;25-27*). A mutant that substantially alters any one of these events, as measured *in vitro*, can have dramatic effects on the proteins ability to function *in vivo*. TrpR is interesting in the fact that mutations that cause super-repressor activity are not localized to functionally relevant areas of the protein, but rather found throughout the protein structure. Similar biophysical characterizations have been carried out on the charge change mutants and have found analogous differences in alpha helical content, protein stability, protein-protein, and protein-ligand interactions, relative to the wild-type protein (*28*). The flexibility of the DNA binding domain is also important to the function of TrpR. The work of Sigler and coworkers have shown that L-trp binding caused changes in the orientations of helix D and helix E in TrpR crystal structures (*3*). It was then speculated that the translocation event of helices D and E was critical for operator DNA recognition in the TrpR system. Later investigations by Lawson et al

(29) revealed that WT-holo-TrpR can adopt two crystal forms, which correspond to two different conformations of the DNA binding domain. This result also indicates that the DNA binding region is quite flexible. NMR has shown that mutations can influence the dynamics of the DNA binding domain which affect function of the protein. The dynamics of the A77V protein have been probed by amide exchange study and revealed backbone exchange rates in the DNA binding domain of A77V are significantly less than rates of holo-WT-TrpR (30). DNA binding studies have shown that the A77V mutant cannot recognize the full complement of operator sequences normally accessible to WT-TrpR (31). Together these results have indicated that the dynamic features of the TrpR DNA binding domain are a critical source of adaptability and allow the protein to recognize a range of operator sequences, while maintaining the ability to reject closely related DNA targets. This is in contrast to the view implied by crystallographic studies which suggested only the orientations of helices D and E were important for operator binding. It is now understood that control of gene regulation within the TrpR system is accomplished through the coupling of multiple equilibria events. Only by the careful dissection of structure and molecular reactions within the repressor protein, have the factors governing gene control in the TrpR system been made clearer.

Due to the potential of temperature-sensitive mutants, (ts), to yield additional information about the relationship between TrpR structure, stability, and dynamics, a genetic selection for such mutants was performed. The selection process was based on the compound 5-methyltryptophan (5-MT), an analog of L-trp, that binds to apo-TrpR about two times more tightly (32), with the resulting 5-MT/TrpR complex binding to

operator DNA about ten times more tightly (33;34). Furthermore 5-MT cannot substitute for the amino acid L-tryptophan during protein synthesis. As a result cells which contain functional TrpR will starve for L-trp on minimal media containing 5-MT, while cells lacking a functional TrpR survive because the operon that controls L-trp production is not repressed (35;36). However after mutagenesis, transformants that grow in the presence of 5-MT are believed to contain inactivating mutations in TrpR (35). Recently Jin et al (35) discovered a (ts) mutant of TrpR, which was selected for growth at 42 °C in the presence of 5-methyltryptophan, and screened for altered growth at 37 °C. This mutation was found to contain a single point mutation at position 75 at the C-terminus of the first helix of the helix-turn-helix motif, with leucine 75 replaced by phenylalanine, and was labeled L75F TrpR (35). To verify that the ts phenotype was due only to the mutation at position 75, the coding sequence for L75F was subcloned into vectors that produced non-regulated levels of the mutant protein. The resulting plasmids were transformed into cells lacking the TrpR gene and displayed the same ts phenotype, growth at 42 °C with weak growth at 37 °C. The results indicated that the L75F mutant has a temperature-sensitive function which is better at 37 °C than at 42 °C. Extensive biophysical and biochemical characterizations revealed that the apo-form of L75F TrpR exhibits an apparent increase in α-helicity as measured by CD, and has a slightly higher urea denaturation mid-point. Fluorescence spectra indicated a more buried environment for one or both tryptophan residues (Trp19 and Trp99) of the mutant protein (35). This data was corroborated via NMR by detection of slower proton-deuterium exchange rates for the spectrally resolved indole ring protons of the

two tryptophan residues in L75F compared to wild-type aporepressor (*35*). Preliminary 1D and 2D-^{1}H NMR spectra indicated that L75F has a 3D structural fold that is very similar to that of WT- TrpR. It was shown that L75F binds L-trp about ten times weaker than wild type TrpR, and the resulting mutant complex binds DNA only about two to five times weaker. Taken together, these data suggested that the leucine to phenylalanine mutation at position 75 generates non-local effects on the dynamics of the protein, with minor consequences on function (*35*).

The molecular mechanism by which the point mutation of a single surface solvent-exposed loop position can lead to global changes in the TrpR protein is still unclear at present. In order to better quantify these non-local effects and their relationship to the structure and/or amide backbone dynamics of the mutant protein, an intensive NMR based study of the mutant TrpR protein L75F has been performed. This work reports the result of a three-dimensional solution structure determination of apo-L75F TrpR derived through NMR analyses. Given the importance of flexibility within the TrpR protein, and the possible long-range effects that the L75F mutation confers on dynamics, the backbone motions of the mutant TrpR protein were also investigated. The backbone dynamics of apo-L75F were derived from ^{15}N relaxation measurements using similar methodology that was originally used with holo-TrpR by Zheng et al (*12*). To quantify dynamical changes of backbone motions of the mutant, this work also investigated the backbone motions of apo-TrpR by ^{15}N relaxation analysis. This was due to the fact that dynamical analysis by ^{15}N relaxation has not been reported on the wild-type apo-TrpR protein and was needed for comparison with apo-L75F. The results

from the structural and backbone dynamics analyses of apo-L75F are presented and interpreted in terms of the known TrpR paradigm. These results are also used to speculate about how modifications within the mutant protein may affect properties which could explain the temperature phenotype.

Due to the chemical-shift overlap of proton resonance signals observed in 1D and 2D ^{1}H NMR spectra for a protein the size of TrpR (25kDa), it was necessary to employ heteronuclear NMR spectroscopy to facilitate resonance assignment. Heteronuclear NMR spectroscopy can aide in resonance assignment of highly overlap proton resonances provided the protein can be labeled with the non-radio active isotopes ^{15}N and ^{13}C. The spectral resolution of highly overlapped ^{1}H signals is improved by increasing the dimensionality of the NMR spectrum so that these problem areas are separated in 2D, 3D, and 4D spectra according to the better resolved heteronuclear resonance (i.e ^{15}N and ^{13}C) (*37*). The apo-L75F protein was labeled with ^{15}N/^{13}C isotopes and the structure was solved using structural restraints derived from conventional heteronuclear (^{1}H, ^{15}N, ^{13}C), multidimensional (2D, 3D, and 4D) solution NMR methods. Structural calculations were performed using the two programs Crystallography & NMR Systems (CNS) (*38*) and X-PLOR version 3.1 (*39*). Determination of the 3D structure of the L75F dimer was based on a total of 1538 ^{1}H nOe-based distance restraints (i.e. 769 unique nOe restraints/monomer), 68 dihedral angle restraints, and 62 hydrogen bond restraints, and resulted in an ensemble of 20 low energy conformers with an average root-mean-square deviation (rmsd) of 1.0 Å for

backbone atoms in the core helices A, B, C, and F, and an average rmsd value of 2.6 Å for helices D and E.

In the course of this work it was discovered that significant ^{1}H-^{15}N chemical shift changes had occurred for backbone amides in L75F when compared to the corresponding chemical shifts reported for wild-type apo-TrpR. It was hypothesized that ring-current effects introduced by the mutant residue phenylalanine may influence nearby amide groups and cause the chemical shift differences. The merit of this hypothesis was addressed by considering the physical nature of the ring-current effect (40) within the context of twenty calculated solution structures of apo-L75F. The data analyses suggest that such chemical shift changes cannot be accounted solely on the basis of ring current shifts originating from the leucine to phenylalanine substitution at position 75. Thus, structural and dynamical differences between apo-L75F and apo-WT-TrpR have been investigated in order to uncover a possible explanation for these non-local effects observed in the mutant protein.

Backbone ^{15}N relaxation measurements have been obtained for 54 residues of apo-WT TrpR, and 75 residues of apo-L75F. The difference in number of residues is due to significant resonance overlap observed in both proteins as well as incomplete resonance assignment available for apo-WT TrpR. Using the structures of apo-L75F and wild type apo-TrpR (5), along with the ^{15}N relaxation data, the motional anisotropy was determined from their respective diffusion tensor. The diffusion tensors for both wild type apo-TrpR, and apo-L75F were independently modeled as axially symmetric prolate ellipsoids when fitted to the ^{15}N relaxation data collected in this study. The anisotropy

was calculated from the principal components of the diffusion tensor ($D_{xx}=D_{yy}=D\perp$, $D_{zz}=D_\parallel$) from the ratio $D_\parallel/D\perp$. This resulted in a value of 1.20 ± 0.04 for apo-L75F and a value of 1.15 ± 0.04 for the apo-WT TrpR. The structural regions of both proteins display similar relaxation signatures, and there are no significant differences regarding the diffusion tensor values $D_\parallel/D\perp$, which reflects the motional anisotropy. Once the diffusion tensors were established for both wild-type and mutant proteins, motional dynamics of backbone NH groups were derived and compared. Models of backbone motions within the context of an axially symmetric diffusion tensor were fit to ^{15}N relaxation data using the program NORMAdyn. The results from this analysis describes motions of NH bond vectors in terms of an order parameter and time scale of motion, similar to the study of Zheng et al (*12*). Due to the complexity of ^{15}N relaxation analysis, a detailed description of the theory and methodology of these types of experiments will be presented in Chapter 2 (Material and Methods) of this thesis. However results from these analyses indicate very similar ^{15}N relaxation profiles for both proteins and do not point to any major dynamical differences within the backbone motions. It should be noted that this work does not rule out the possibility that other dynamic differences may exist between apo-L75F and apo-WT-TrpR. Side chain dynamics obtained by ^{13}C relaxation studies or probing the slower millisecond timescale may lead to insightful contrasts between the two proteins that would help explain the effects of the L75F mutation.

The NMR study presented herein supports the biochemical and biophysical findings of Jin et al (*35*), that the leucine to phenylalanine mutation at position 75

indeed propagates long-range effects throughout the protein. This work quantifies structural and dynamical differences at the atomic level of apo-L75F by comparison with apo-WT TrpR under identical solution conditions. It is shown that hydrophobic core of apo-L75F has undergone a small perturbation. This is reflected in the distances between helices C/C', helices A/C', and helices C/F relative to corresponding values obtained from the published structures of apo-WT TrpR *(5)*. Chemical shift differences, together with inspection of the apo-L75F TrpR structures, also point to a reordering of the L-Trp binding pocket in the mutant protein. Overall the ^{15}N relaxation data probing the NH backbone dynamics do not seem to indicate major differences between the apo-forms of wild-type and mutant proteins. The only exception is that simpler models of backbone motions can be used to fit NH groups within the DNA binding domain of apo-L75F and may suggest a decrease in motional complexity of this region relative to the wild-type apo-protein. This analysis suggests that if major dynamical differences exist between the two proteins, they may manifest on a slower microsecond to millisecond time scale, or may be reflected in dynamical differences of sidechains.