4-24-2003

Characterizing Conformational Changes Along the Dimerization Helix of the Global Regulator, FNR, Using Fluorescence Spectroscopy

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Abstract

The FNR protein is a transcription factor that allows *Escherichia coli* to undergo anaerobic cellular respiration. It is known to positively regulate the expression of several genes required for anaerobic respiration as well as negatively regulate genes responsible for aerobic respiration. Consequently, FNR is active under anaerobic conditions and inactive under aerobic conditions. Although the tertiary structure of FNR is unknown, previous studies have indicated that FNR is inactive in the monomeric state and active in the dimeric state. Thus, it is believed that in anaerobic conditions, FNR undergoes a conformational change from the monomeric to dimeric state. The mechanism involved in going from the monomeric to dimeric state is not completely understood, but it is thought to be triggered by the acquisition of a $[4Fe\text{-}4S]^{2+}$ cluster in the N-terminal region of FNR. The acquisition of the cluster causes a conformational change to be transmitted through the allosteric domain to the dimerization helix resulting in the active dimeric species. Information regarding the environment of amino acid residues in the dimerization helix in both the active and inactive forms of FNR could be helpful in eliciting a better understanding of the dimerization mechanism. Such environmental conditions can be determined by the fluorescent properties of the amino acid, tryptophan. Surface exposed tryptophan residues are expected to have a longer λ_{max} than those buried in the hydrophobic core. In order to gain insight into the environment of the amino acids on the dimerization helix we have created tryptophan mutants (LW146, MW147, MW157, KW163, and KW164) that either lay on or near the helix. The mutants KW163 and KW164 all lie on the periphery of the helix while LW146, MW147 and MW157 lie on the helix. Of the five mutants, KW163 retained anaerobic activity most similar to that of

the wild type indicating that its structure is similar to the wild type protein with the exception of the single amino acid substitution. By comparing the fluorescence of the active and inactive forms of KW163, we hope to gain a better understanding of the dimerization mechanism of FNR.

Introduction

FNR as a Transcription Factor in Anaerobic Respiration

Transcription factors activate or repress the transcription of specific genes to regulate protein levels in a cell. Repression occurs through either blocking RNA polymerase (RNAP) from binding to the promoter region of a gene, or preventing elongation along the DNA template strand. Activation involves either direct or indirect contact with RNAP that facilitates binding of RNAP to the promoter region. Because of their regulatory role in gene expression, the synthesis and activity of transcription factors is a key element in responding to changing cellular environments. In *Escherichia coli,* one such environmental change is oxygen deprivation that leads to a switch from aerobic respiration to anaerobic respiration. Not all organisms can respire anaerobically, and like aerobic respiration, anaerobic respiration involves a complex series of regulatory enzymes. Under anaerobic conditions, molecular oxygen is no longer available to serve as the final electron acceptor in cellular respiration. Consequently, the cell must utilize alternate electron acceptors such as nitrate, nitrite, fumarate, and dimethysulfoxide (DMSO) (1). As can be seen in Table 1, different final electron acceptors differ in their reduction potentials. Consequently, the final electron acceptor used will depend upon availability and reduction potential. The cell will preferentially use those acceptors with greater reduction potentials to give higher energy yields.

Table 1: Oxidation-Reduction Potentials (E_o') of Various Final Electron Acceptors in Catabolic Pathways

Final Electron Acceptor	*E_o' (mV)*
Oxygen	+820
Nitrate	+420
Nitrite	+374
DMSO	+160
Fumarate	+31

Values obtained from Ref. 1

In the facultative anaerobe, *Escherichia coli*, the transcription of the genes encoding enzymes necessary to reduce various final electron acceptors are aided by the transcription factor, FNR. Studies have shown FNR to be essential for the transcription of nitrate reductase (*narGHJI),* NADH-dependent nitrite reductase (*nirBDC),* and necessary for maximal activation of fumarate reductase (*frdABCD)*, and DMSO reductase (*dmsABC)* (1). FNR also acts as a suppressor of genes encoding for factors necessary in aerobic respiration such as the pyruvate dehydrogenase complex (*pdh),* NADH dehydrogenase (*ndh),* and Mn-Superoxide dismutase (*sodA)* (1). As expected from its function, FNR becomes active under anaerobic conditions and inactive under aerobic conditions. Thus, the activity of FNR is mediated by the presence of molecular oxygen.

Although FNR is the primary regulator during anoxia, it interacts with other transcription factors to ensure that anaerobic respiration utilizes the path yielding the greatest energy. For example, NarL regulates genes in response to nitrate and

anaerobiosis. Studies have shown that FNR activation of *narG* is enhanced in the presence of nitrate, and reduced in *narL* mutants (1). This suggests that NarL enhances transcription of *narG* by interacting with FNR. However, NarL does not always enhance FNR activity. It serves the antagonistic role of a repressor at *dmsA* (1). This suggests that NarL functions independently of FNR to ensure that the cell undergoes the most energy efficient means of anaerobic respiration.

FNR Structure and its Homology With CAP

FNR belongs to a larger family of global regulators that includes the catabolic activating protein, CAP also referred to as CRP (cAMP receptor protein). CAP is responsible for facilitating and repressing the transcription of a number of genes involved in utilizing appropriate carbon sources for catabolism, and is the major transcriptional activator of the *lac* operon (2). CAP exists as a dimer and is activated when glucose levels decrease in the cell via an interaction with the effector molecule, cAMP (2). The binding of cAMP triggers a conformational change in CAP allowing it to bind to a regulatory element in the *lac* operon. CAP, shown in Figure 1, is characterized by a helix-turn-helix DNA binding domain located at the C-terminal region, and a larger β-roll N-terminal domain comprised of a dimerization helix and nearby cAMP binding site (3). CAP also contains three surface exposed activating regions (AR1, AR2, and AR3) that interact with RNAP(4). At Class I promoters (CAP/FNR binding site at approx. -61.5[1]), such as the *lac* operon, interaction between CAP and RNAP is mediated by contacts between AR1 on the downstream subunit and αCTD of RNAP (4). At Class II promoters

[1] The Class I binding site at –61.5 refers to a position that is 61.5 basepairs upstream from the start of transcription.

(-41.5, see Figure 2), AR1 is functional on the upstream subunit while AR2 is functional on the downstream subunit. AR1 and AR2 interact with RNAP at the αCTD and αNTD respectively (5). AR3 is located on the downstream CAP subunit and interacts with the σ subunit of RNAP to directly facilitate DNA binding (5).

Sequence homology between CAP and FNR has made the structure and function of CAP a valuable model for investigating FNR. Although the tertiary structure of FNR has not been determined, a theoretical structure based on the known structure of CAP has been proposed (Figure 3). The most striking similarity between CAP and FNR is the C-terminal α-helix-turn-α-helix DNA binding domain. FNR binds to a 22 base pair region containing a 5 base pair palindromic sequence (TTGAT X_4 ATCAA) so similar to that of CAP that a three base pair substitution is sufficient to allow FNR activation of the *lac* promoter (6, 7). FNR also is thought to contain a β-roll allosteric domain analogous to the cAMP binding domain of CAP. This domain in FNR is thought to have a dimerization helix similar to the one found in CAP (3, 8, 9). Like CAP, FNR also has three activating regions (AR1, AR2, and AR3) that have been confirmed through mutational analysis of FNR to make contact with RNAP. AR1 of FNR is homologous to that of CAP and functions at both Class I and Class II promoters through interactions with the αCTD of RNAP (10). AR3 is analogous to the AR3 of CAP and functions in the downstream subunit of the FNR dimer to regulate transcription via interactions with the C-terminal domain of the σ subunit of RNAP (5). In the FNR protein, AR2 is normally inactive except to compensate for an impaired AR1 (10).

A small region in the N-terminus of FNR (residues 1-29) was found to have no corresponding region in CAP. Mutational analysis has shown that three of the four

cysteine residues in this region (C20, C23, C29) as well as C122 in the allosteric domain are essential for the *in vivo* functioning of FNR (9). It was hypothesized that these residues functioned as metal ligands, and subsequent spectral analysis identifying a [4Fe-4S]$^{2+}$ cluster in the active form of FNR supported this hypothesis (7, 8, 9). FNR is known to be active as a dimer and inactive as a monomer. Thus, it is believed that anaerobic conditions shift FNR from an inactive monomer to an active dimer. Although the exact mechanism of the monomeric-dimeric shift is not known, it is believed that under anaerobic conditions the acquisition of a [4Fe-4S]$^{2+}$ cluster causes a conformational change to be transmitted through the allosteric domain and into the dimerization helix resulting in the formation of the active dimer (7, 8, 9). The CAP model provides no information as to the dimerization mechanism in FNR because CAP exists as a dimer whether or not it is active.

Despite a lack of information regarding the conformational changes that occur during dimerization of FNR, a dimerization helix has been characterized by previous studies involving alanine mutations (11). Positions 140-159 in FNR are thought to be analogous to CAP residues that stabilize the active dimer through coiled-coil interactions (11). Alanine substitutions in positions 140-159 identified a series of hydrophobic residues essential for FNR to be functional *in vivo*. A decrease in FNR activity of less than fifty percent wild type was observed upon replacing the residues R140, M143, M144, L146, M147, I151, M157, and I158[2] (11). The hydrophobic nature of many of these residues coupled with their spacing 3-4 residues apart strongly supports the notion that FNR also dimerized via coiled-coil interactions (11). It was proposed that residues R140, M144, M147, I151, and I158 composed a dimerization interface where

[2] All amino acids have three letter and one letter abbreviations that are listed in Appendix 1.

hydrophobic interactions between two inactive monomers would promote the formation of an active dimer (11). Understanding the local environmental changes that occur along the proposed dimerization helix could result in a better understanding of the conformational changes associated with dimerization in FNR.

Fluorescence

Information regarding the environment of the dimerization helix in both the monomeric and dimeric form of FNR can be obtained through a combination of site –directed mutagenesis and fluorescence spectroscopy. Fluorescence is dependent upon the excitation and subsequent relaxation of a molecule upon absorption of a photon. The absorption of a photon by a molecule results in the ejection of an electron from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) where it exists as a singlet (12). HOMO and LUMO are commonly referred to as the ground state (S_0) and excited state (S_n) respectively and are represented in Figure 4. The energy difference between these two states is equal to the energy of the photon which can be determined by Eq. 1:

$$E = hc/\lambda \qquad (1)$$

with h being Planck's constant (6.6256×10^{-34} Js), c being the speed of light (3×10^8 m/s), and λ being the wavelength of light.

Each excited state contains a set of vibrational energy states (V_n), and the excited electron is usually ejected to a higher vibrational state than the V_0 of S_n. Thus, initially an excited electron generally exists in the V_n of S_n. Upon reaching this level, the electron

will immediately drop down to the most stable vibrational state (V_0) of S_n with the energy difference being dissipated as heat. The electron will follow the same pattern of relaxation until it reaches the highest vibrational energy state (V_n) of the most stable excited state (S_1). At this point any further relaxation can occur by a number of different mechanisms outlined in Figure 4:

1. Intersystem crossing (ISC) is characterized by the excited electron undergoing a spin inversion resulting in a triplet state of lower energy due to the parallel spins of the original electron pair. Intersystem crossing is rare and energy dissipated this way is represented as $E_{ISC}(12)$.

2. Photoproduct formation (PR) occurs when the excitation energy at the S_1 state results in a change in the overlap of molecular orbitals that is conducive to the formation of new bonds resulting in a new molecule referred to as the a photoproduct with an associated energy defined as $E_{PR}(12)$.

3. Non-radiative (NR) deactivation occurs when V_0 of S_1 overlaps with the highest vibrational level (V_n) of S_0. If this is the case, the electron will move to V_n of S_0 and drop down to V_o dissipating the energy differences as heat in the same manner as described for the descent from V_0 of S_n to V_n in S_1. The resultant energy change is termed $E_{NR}(12)$.

4. Quenching is the absorption of energy from the excited electron by another solute molecule appropriately termed the quencher and will be examined in more detail later in reference to its affects on protein fluorescence. E_Q is defined as the energy dissipated through quenching (12).

5. Radiative deactivation or fluorescence is defined as the return of the excited electron to any vibrational state in S_0 accompanied by the emission of a photon equal in energy to the difference between the two energy levels (12). The energy emitted through fluorescence is represented by E_F and is represented by Figure 4 as *hv*.

Intersystem crossing, photoproduct formation, and nonradiative decay are not a direct concern to this study, but they do have effect on fluorescence. Due to the first law of thermodynamics, the energy absorbed will be equal to the energy given off during relaxation and be of a constant value. Consequently, all methods of relaxation are in competition and can be represented by Eq. 2:

$$E_{emission} = E_{ISC} + E_{PR} + E_{NR} + E_Q + E_F \qquad (2)$$

For this relation to hold true an increase in one E value is reflected in a corresponding decrease in another. Thus, the relaxation due to fluorescence is dependent upon the competing methods of relaxation which are directly related to the environment of the fluorophore. However, when a macromolecule undergoes a conformational changed, it can be assumed that EISC, EPR, and ENR remain constant. Thus, the only energy changes observed will be those due to quenching and fluorescence. The change in fluorescence can be an extremely useful biological tool especially in obtaining information regarding the structure of macromolecules. .

Amino Acids as Environmental Probes

In proteins, the amino acid residues phenylalanine, tyrosine, and tryptophan all have intrinsic fluorescent properties and can thus be used as fluorescent probes. Because fluorescence is dependent upon absorption, these residues can be classified based on their ability to absorb photons according to the Beer-Lambert Law (12):

$$A = \varepsilon cl \qquad (3)$$

where c is the concentration of the amino acid in solution, l is the length light travels through the spectrophotometer cuvette, ε is the molar absorption coefficient which is an intrinsic property of the fluorophore representing the absorption of a one molar solution with an l of 1 cm (mol^{-1} cm^{-1}). It follows from this relation that A is directly proportional to ε.

As previously predicted by Eq. 2, the amount of energy emitted through fluorescence is directly influenced by the alternate methods of energy dissipation. Consequently, the ratio of photons emitted to photons absorbed can be represented by Eq. 4 (12):

$$\Phi_F = K_F/[K_{ISC} + K_{PR} + K_{NR} + K_Q(Q) + K_F] \qquad (4)$$

where Φ_F represents the quantum yield and K represents the rate constant of the respective competitive processes of de-excitation. Both the quantum yield and ε give

valuable information regarding the experimental sensitivity of a fluorophore and can be related by Eq. 5:

$$S = (\Phi_F)(\varepsilon) \qquad (5)$$

Table 2: Fluorescent Properties of Potential Amino Acid Probes

Fluorphore	ε_{max} ($x10^{-3}$)	Φ_F	**Sensitivity** ($x10^{-2}$)	**Abs.** λ_{max} **(nm)**	**Emission** λ_{max} **(nm)**
Tryptophan	5.6	0.20	11	280	348
Tyrosine	1.4	0.14	2.0	274	303
Phenylalanine	0.2	0.04	0.08	257	282

Values obtained from Ref. 13

The experimental sensitivity is a key component to measuring the effectiveness of a fluorescent probe. A probe with greater sensitivity will be a more useful tool in obtaining relevant biological information such as the immediate environment surrounding an amino acid.

Although phenylalanine, tyrosine, and tryptophan all have intrinsic fluorescent properties they differ greatly in their ability to act as probes. Table 2 indicates the molar absorption coefficients, quantum yields, sensitivities, absorption λ_{max} and emission λ_{max} of the three amino acids based on measurements taken of free amino acids in H_20 at pH = 7.

Based on the sensitivity values in Table 2, it can be seen that tryptophan is the most responsive fluorescent probe of the three while the extremely low value of phenylalanine usually results in its being ignored as a contributor to observed

fluorescence. However, experimental sensitivity is not the only reason tryptophan is so useful. Although the number of tyrosine residues in a protein is generally small, the number of tryptophan residues is even less . Therefore, the fluorescence observed due to tryptophan can be more accurately attributed to a specific region in the protein.

One potential problem in analyzing proteins with both Trp and Tyr residues is that the maximum absorbance of both amino acids is 280 nm. Consequently, determining whether or not fluorescence is due to Trp or Tyr can be difficult. This is partially avoided by using an excitation wavelength of 295nm. Tryptophan's greater molar absorption coefficient relative to Tyr allows absorption to occur over a wider range of λ_{ex}. At 295 nm, value of ε of Tyr is nearly zero as seen in Figure 5. Therefore, fluorescence observed from excitation at this wavelength can be attributed to Trp. If, however, the number to Tyr residues present makes a substantial contribution to fluorescence at 295 nm, the excitation wavelength can be shifted to 300 nm. In addition to altering the excitation wavelength to preferentially excite Trp, resonance energy transfer from Tyr to Trp also serves to minimize Tyr fluorescence. Resonance transfer can occur when the emission wavelength of a potential donor overlaps with the absorption wavelength of a potential acceptor in close proximity within the protein. When this occurs, the energy can be transferred from the donor to the acceptor. Values in Table 2 indicate that resonance energy transfer is possible from Tyr to Trp. Consequently, the energy absorbed by Tyr can ultimately contribute to the fluorescence of Trp if that Tyr residue(s) is in close proximately to Trp. This phenomenon can give valuable information regarding the position of Tyr and Trp residues relative to one another.

The actual intensity and wavelength of maximum emission of Trp fluorescence is an important indictor of its local environment. Interactions with solvent molecules as well as intramolecular interactions with cysteine, histidine, asparagines, and glutamine can all result in decreased intensities and blue/red shifts in the maximum emission wavelength due to energy transfer processes. Our focus will mainly be solvent interactions. A surface exposed Trp residue will fluoresce at a lower energy and therefore greater wavelength than a residue buried in the hydrophobic core of a protein. Conversely, a residue buried in the interior of a protein will be blue shifted relative to one on the solvent exposed surface. In general, tryptophan in proteins will fluoresce between 320 nm to 350nm although values as low as 308 nm have been recorded (14).

Using Tryptophan Fluorescence to Analyze the Conformational Change of FNR

The purpose of this study is to utilize the fluorescence of tryptophan as seen in Figure 6 to gain a better understanding of the dimerization mechanism of FNR. Because FNR contains no tryptophan residues, single amino acid substitutions were carried out to create the five mutant proteins: LW146[3], MW147, MW157, KW163, and KW164 (Figure 7). The substitution sites were selected based on their proximity to the dimerization helix with LW146, LW147, and MW157 lying within the helix while KW163 and KW164 are located just on the periphery in a hinge region. If these mutants resemble wild type activity under anaerobic conditions and are inactive like the wild type under aerobic conditions, it can be inferred that they are similar in structure to the wild type protein with the exception of the amino acid substitution. It follows then that the mechanism of

[3] A standard site-directed mutagensis code is used where the first letter stands for the amino acid in the wild type and the second letter is the amino acid in the mutant. LW146 refers to a tryptophan residue being inserted in place of a leucine residue at position 146.

dimerization will be similar in all five mutants as it is in the wild type. Consequently, comparing the fluoresce intensity of the purified mutants in anaerobic conditions to that of aerobic conditions should yield valuable information regarding the local environmental changes that occur along the dimerization helix during activation of FNR.

Potential problems in this study include the possibility of attributing tyrosine fluorescence to tryptophan and quenching by molecular oxygen in the aerobic experiment. Since FNR contains only five tyrosine residues (Figure 7) this first concern is minimized because only five tyrosine residues are present. Therefore, excitation at 295 nm should be sufficient to preferentially select for tryptophan fluorescence. The latter concern may pose a more serious concern. Oxygen has been shown to be small enough to diffuse into the core of a protein. Therefore, the presence of oxygen can cause a decrease in fluorescence intensity possibly resulting in the inability to determine the environmental condition of the tryptophan residue (14). This could become problematic in studying conformational changes when FNR goes from the anaerobic active form to the inactive aerobic form. If oxygen quenching occurs, then differences between the two spectra cannot conclusively be attributed to conformational changes. Determining whether or not oxygen quenching will occur can be addressed using a control experiment with the previously isolated mutant LH28. LH28 has been previously characterized as being active under both aerobic and anaerobic conditions because the $[4Fe\text{-}4S]^{2+}$ is not sensitive to oxygen (8). Because of its apparent oxygen insensitivity, the anaerobic conformation of LH28 is thought to be the same as the aerobic conformation. Thus, the fluorescent spectra of the aerobic and anaerobic protein should be identical. Any deviations can be attributed to quenching that might occur during oxygen exposure.

Materials and Methods

Growth Conditions and Parental Strains

The *E. coli* strains used in this study are listed in Table 3 along with any relevant information regarding their respective genotypes and general usage. Unless otherwise indicated, all strains were grown in LB at 37°C with ampicillin at a concentration of 50μg/ml.

TABLE 3: Strains and Plasmids

Strain	Genotypic Information	Usage
RZ1032	*dut⁻ ung⁻* (15)	Synthesis of single stranded DNA for mutagenesis
DH5α	*dut⁺ ung⁺ recA⁻ endA⁻* (15)	Mutagenesis and cloning
RZ8480	Φ(*narG-lacZ*), Δ*fnr* (6)	Activity assay
PK22	Δ*cap*, Δ*fnr* (6)	Protein purification
RZ3293	Φ(*dms-lacZ*), Δ*fnr* (11)	Activity assay

Plasmids and Vectors	Genotypic Information
pPK821	Amp^r, HindIII - *Bam*H1 of wild type *fnr* at -521 and +1115 in pUC118 with an *Nde*I site at +1 (6)
pET11a	Amp^r, T7*lac* promotor and gene *10* translation start directly upstream from *Nde*1 site (6)
pPK823	Amp^r, *Nde*1 - *Bam*H1 of pPK823 (+1 to +1115) in pET11a (6)

Mutagenesis

The initial mutagenesis of the FNR protein was carried out on pPK821 (see Table 3) using oligonucleotide-directed mutagenesis using single stranded DNA with T4 DNA polymerase and T4 DNA ligase. Oligonucleotide-directed mutagenesis consists of three general steps in which a synthetic primer coding for the desired mutation is annealed to the target region of the single stranded wild type gene to initiate *in vitro* DNA synthesis, DNA polymerase extends the primer to create double stranded DNA on which one strand has the desired mutation and the other is the wild type strand, DNA ligase seals gaps bordering the newly synthesized strand, and finally the newly synthesized double

stranded DNA is transformed into a strain of *E. coli* and allowed to grow on media plates. The plasmid DNA is isolated from resulting bacterial colonies and sequenced to check for the presence of the desired mutation (15). The products of oligonucleotide mutagenesis will be fifty percent wild type and fifty percent of the desired mutation due to the wild type template strand that does not contain the mutation. However, selection for the mutant strand was enhanced using the Kunkel Method (15). The Kunkel Method takes advantage of an *E. coli* strain's uracil-DNA glycosylase that will destroy DNA that has uracil incorporated instead of thymine. Starting with single stranded DNA that contains uracil and synthesizing a mutant strand *in vitro* that contains thymine followed by a transformation into a strain that contains uracil-DNA glycosylase (ung^+) will result in the destruction of the wild type template strand and a far greater yield of the desired mutant (15). In this study, the single stranded wt *fnr* template strand was synthesized from pPK821 (*fnr*/pUC118) transformed into the RZ1032 strain (dut^- ung^-). RZ1032 was grown in 2XYT media supplemented with Vitamin B_1 (0.01mg/mL) and ampicillin at a final concentration of 50μg/mL. The culture was then infected with the M13K07 helper phage to produce single stranded DNA from pPK821. The resultant wild type single stranded pPK821 was used to synthesize the desired mutants *in vitro* with T4 DNA polymerase and T4 DNA ligase. Table 4 lists the primers used corresponding to the appropriate mutant with bold lettering indicating the insertion site of a tryptophan residue. Primers were synthesized by Integrated DNA Technology (IDT). Products were transformed into DH5α cells using 0.1 M $CaCl_2$. Plasmid DNA from resultant bacterial colonies was isolated using Qiagen Mini-prep Kit. Mutants were determined by sequencing using SequiTherm Excel II Sequencing Kit.

TABLE 4: Primers Used in Site Directed Mutagenesis

Plasmid	Wild Type Sequence and Mutagenic Primer
WT	GCAGAUGAUGCGU**CUG**AUGAGCGG
LW146	GCAGATGATGCGT**TGG**ATGAGCGG
WT	GCGUCUG**AUG**AGCGGUGAA
MW147	GCGTCTG**TGG**AGCGGTGAA
WT	GAUCAGGAC**AUG**AU**C**CUGCUGUUGUCG
MW157*	GATCAGGAC**TGG**AT**A**CTGCTGTTGTCG
WT	CUGUUGUCG**AAG**AAAAAUGCC
KW163	CTGTTGTCG**TGG**AAAAATGCC
WT	GUUGUCGAAG**AAA**AAUGCCGAGG
KW164	GTTGTCGAAG**TGG**AATGCCGAGG

**A second, silent mutation was made in MW157 at position I158 (AUC – ATA) to avoid the insertion of a second BamH1 site.*

Constructing FNR/pET11a Plasmid

The mutant *fnr* genes were inserted into pET11a. pET11a is a low copy number plasmid with a *T7lac* promoter to allow for tighter control of transcription. Plasmid construction utilized the *Nde*1 and *Bam*H1 restriction endonuclease sites flanking the *fnr* gene in pUC118 at positions +1 and +1115 respectively to the transcription start site and present in pET11a at base positions 359 and 319. The mutated *fnr* gene was excised from pUC118 using a two step process. The *fnr* gene was digested sequentially with *Bam*H1 and *Nde*1. Preparations of pET11a proceeded along the same lines. The appropriate DNA fragments were then isolated from a 1% agarose gel and purified with Prep-a-Gene DNA purification kit from BioRad. The purified *fnr* gene and pET11a vector were then ligated together using a 2:1 ratio of *fnr* : pET11a and 1μL DNA ligase in an 10μL final volume. The ligations were transformed into DH5α and plasmid DNA from resultant colonies was isolated using Qiagen Mini-prep Kit . Correct ligation

products were determined through size comparison of the ligated plasmids to pET11a and pPK823 on a 1% agarose gel. Ligation products matching the size of pPK823 (wt *fnr*/pET11a) were deemed correct. Gel results were later verified by DNA sequencing at the University of Wisconsin-Madison.

Activity Assay

LW146/pET11a, MW147/pETl11a, MW157/pET11a, KW163/pET11a, and KW164/pET11a were all transformed into RZ8480 or RZ3293 and grown up anaerobically in 10mL glucose minimal media with the addition of ampicillin at 50μg/mL. Media for assays involving RZ8480 were supplemented with 1mM KNO_3. Activity was measured using the β-galactosidase assay and o-nitrophenol galactose (ONPG) at 4mg/mL. Cells were grown such that optical density at 600nm and did not exceed 0.8. The β-galactosidase reactions were terminated with 0.5mL of 1M Na_2CO_3 and the absorbance was measured at 550 nm and 420 nm. Aerobic assays were also performed on MW147/pET11a, pPK823, and KW163/pET11a in RZ8480 using the same procedure. Aerobic cultures were grown up in 3mL glucose minimal media with ampicillin at 50μg/mL. The optical density at 600 nm was not allowed to exceed 0.3 in these aerobic cultures.

Protein Purification

Wild type FNR and LH28 were purified at the University of Wisconsin-Madison using a previously published procedure (11).

Fluorescence Data

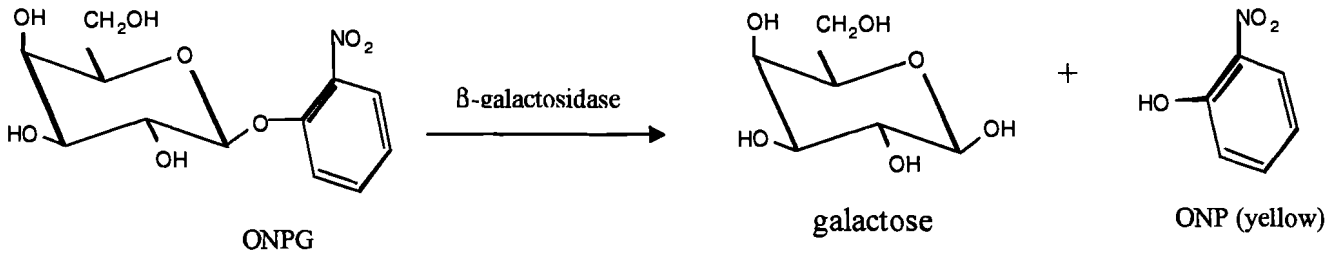
Both aerobic and anaerobic spectra were obtained using Perkin Elmer Luminescence Spectrophotometer LS 50B. Wild type FNR fluorescence was measured in a 400mM phosphate buffer solution pH 6.8 with a final protein concentration of 7.35μM. LH28 fluorescence was measured in a 400mM phosphate buffer solution pH 6.8 at a final protein concentration of 10.5 μM. Aerobic and anaerobic spectra of the wild type FNR and LH28 were obtained with an λ_{ex} = 280 nm and 295 nm. All spectra were obtained with an exit and enter slit of 10nm and a scan speed of 100nm/min.

Results

Activity Analysis of FNR Mutations

The activity of the FNR protein was first measured using a β-galactosidase assay under anaerobic conditions in the RZ8480 strain, in which the *narG* promoter is fused to the *lacZ* gene (*narG-lacZ). NarG* is a class II promoter with an FNR binding site centered at –41.5 from the transcription start site. When active, FNR binds to *narG* to activate expression. Although FNR is required for expression of *narG*, an enhanced expression is observed upon addition of nitrate and the subsequent induction of the transcription factor NarL. Thus, FNR activation of *narG* is enhanced by NarL (1). *LacZ*

codes for the enzyme β-galactosidase which cleaves lactose into glucose and β-galactose for utilization in cellular metabolism. O-nitrophenol galactose (ONPG) serves as an analog to lactose and is used as a substrate for β-galacotosidase. When present, β-galacotosidase will catalyze the conversion of ONPG to galactose and ortho-nitrophenol (ONP) which is yellow in solution as illustrated below:



ONPG galactose ONP (yellow)

In the RZ8480 strain, expression of *lacZ* is dependent upon FNR binding at the *narG* promoter. When FNR is active, *lacZ* will be expressed in the cell and the ONPG conversion catalyzed by β-galactosidase will cause the solution to turn yellow.

Within RZ8480, LW146, MW147, MW157, and KW164 show a significant reduction in activity while KW163 only shows a slight reduction (Table 5).

Table 5: FNR activity of tryptophan mutants in RZ8480

FNR Substitution	Activity reported as % WT FNR (with standard deviation)
fnr⁻	1.7 ± 0.68
LW146	6.7 ± 1.5
MW147	26.7 ± 4.3
MW157	22.9 ± 7.2
KW163	81.1 ± 4.3
KW164	18.9 ± 6.2

Previous studies that found alanine mutations at positions 146, 147, and 157 significantly reduced FNR activity (11). The reduced activity at position 147 in conjunction with its position relative to other mutants with significantly reduced activities suggested that M147 was positioned along the interface of the dimerzation domain where the presence of the hydrophobic residue, methionine, is thought to stabilize the dimerization interface via hydrophic interactions. Inserting an alanine in place of methionine results in a significant decrease in occupied volume along the helix as can be seen in Table 6 while the insertion of a tryptophan increases the occupied volume.

Table 6: Average Volume Occupied by Amino Acids in the Interior of a Protein

Amino Acid	Average Volume ($Å^3$)	Standard Deviation of $Å^3$
Methionine	170.8	9.6
Leucine	167.9	10.2
Alanine	91.5	6.7
Tryptophan	237.6	13.6
Lysine	171.3	6.8
Glutamate	155.1	11.4

Values obtained from ref. 16

This change in volume could destabilize the dimerization interface. This hypothesis is supported by the observation that MA147 purified as a monomer under anaerobic conditions (11). However, the change in volume associated with a tryptophan substitution is not as great. Additionally, tryptophan is relatively hydrophobic (Appendix 1). Thus, the hypothesized cause of the reduction of activity observed in the

alanine mutation cannot be extrapolated with certainty to the tryptophan substitution. The reduction of activity due to substitutions at positions 146, 157 and 164 cannot be attributed to disruptions along the dimerization interface as these residues are not along the proposed interface region. One possible explanation for the reduction in activity observed in LW146, MW147, MW157, and KW164 is that the protein is present in lower concentrations than the wild type. However, previous studies utilizing alanine mutations in pET11a have shown that protein concentrations are equal between the wild type and mutant FNR (11). In contrast to LW146, MW147, MW157, and KW164, KW163 shows only moderate reduction in activity. This finding was surprising considering the reduction in activity in the KW164 substitution which is adjacent to K163 in the same hinge region.

MW147 and KW163 were further tested under aerobic conditions relative to the wild type and the previously isolated mutant DA154 (8) that is active under both anaerobic and aerobic conditions. As expected, MW147 and KW163 and the wild type were inactive (results not shown).

Reduced activity observed in RZ3293 is consistent with RZ8480.

FNR activity was subsequently tested in the *E. coli* strain RZ3293 and shown in Table 7:

Table 7: FNR activity of tryptophan mutants in RZ3293

FNR Substitution	Activity reported as % WT FNR
fnr$^-$	18.0 ± 0.6
LW146	29.2 ± 5.2
MW147	37.2 ± 9.5
MW157	40.8 ± 4.1
KW163	71.7 ± 4.3
KW164	32.9 ± 7.9

Activity tests in RZ3293 were performed to ascertain whether reduction in activity observed in the tryptophan mutants was promoter specific. RZ3293 is similar to RZ8480 except *lacZ* expression is dependent upon FNR binding at the *dmsA* promoter region. Like the *narG* promoter, *dmsA* is a class II promoter with its FNR binding site located slightly upstream at –49.5 compared –41.5 at *narG* (1). In contrast to the severe reduction in activity observed in RZ8480, the effects of the single substitutions were not as marked in RZ3293 although the general trends remained the same. The increased activity of the *fnr*$^-$ strain indicates that the *dmsA* promoter is not as tightly regulated as *narG*. Consequently, increases in activity cannot necessarily be attributed to a functioning FNR.

The Fluorescence Spectrum of WT FNR Indicates Minimal Tyrosine Fluorescence

Fluorescent spectra of wild type FNR were obtained anaerobically and aerobically at 280 nm and 295 nm to determine the fluorescence of the five tyrosine residues present in the wild type protein (Figure 7). Tyrosine has a maximal absorbance at 280 nm. Therefore, in theory, the most significant contribution tyrosine residues would make to the fluorescent spectra would be at an excitation of 280 nm. Any contribution by tyrosine should be dampened at 295 nm so as not to interfere with potential tryptophan fluorescence in the mutant FNR. The emission maximum wavelength of the anaerobic and aerobic forms of FNR at 280 nm was approximately 335 nm is significantly red shifted relative to the expected value of 303 nm of tyrosine free in solution (Figure 8). Within a protein, the maximum emission, in general, should be blue shifted relative to 303 nm. Thus, it appears that the small intensity observed in the wild type at 335 nm is not due to tyrosine fluorescence but rather another protein in the solution. The emission maximum of the anaerobic and aerobic wild type species at 295 nm was approximately 345 nm (Figure 9). This red shift relative to the tyrosine value in free solution (303 nm) further indicates that tyrosine is not contributing to the fluorescent spectra. The emission maximum observed, however, does further support the notion that there is an impurity in the solution. However, the intensity observed is very small indicating that either the impurity is in a small quantity or it has little intrinsic fluorescence. The difference in the intensity between the anaerobic and aerobic forms does not appear to be significant.

The fluorescent spectra of LH28 is inconclusive.

LH28 was used as a control to determine whether or not oxygen quenching would be problematic in comparing the anaerobic spectrum to the aerobic spectrum of the

tryptophan mutants. LH28 is active in both an aerobic and anaerobic environment indicating that it has the same conformation regardless of oxygen concentration (20). Thus, any change in the fluorescence spectrum between the anaerobic and aerobic forms of LH28 can be attributed to oxygen quenching and not conformational changes within the protein. If oxygen quenching were to be problematic we would expect that the intensity of the aerobic form of LH28 would be significantly decreased relative to the anaerobic form. Figure 10 shows this not to be the case at 295 nm. Additionally, there is not a shift in the emission maxima which further suggests the environment of the fluorophore is not changed by oxygen. However, the high intensity observed in both forms of LH28 indicates that another protein in the solution has a significant amount of fluorescence or is present in high concentration. This intensity cannot be attributed to the five tyrosine residues in LH28 because the emission wavelength of 345 nm is uncharacteristic of tyrosine, and the spectra were obtained at 295 nm which should not allow significant tyrosine fluorescence. Results obtained at 280 nm show a similar trend (data not shown). Therefore, the spectra obtained from LH28 is probably not an accurate measurement of LH28 but rather another protein in solution.

Discussion

The effects of tryptophan substitutions appears to be promoter independent.

Activity readings from both RZ8480 and RZ3293 show a reduction in activity for substitutions tested (see Figure 11). The similar changes suggest that the effects of

tryptophan mutations at the respective positions is not promoter specific. Although there is an increase in activity in RZ3293, it can be attributed to basal transcription levels observed in the fnr$^-$ and not the mutations themselves. In other words, the increase in activation observed in RZ3293, with the exception of KW163 is general to all mutations.

KW163 most closely resembles the wild type.

Based on activity readings in RZ8480 and RZ3293, the activity of KW163 most closely resembles that of the wild type. It can therefore be inferred that the structure of KW163 is the closest to wild type relative to the other four mutants. Data obtained by comparing the fluorescence spectrum of the anaerobic and aerobic forms of the isolated KW163 can therefore be used to represent changes in the wild type protein. The activity of KW163 was surprising in light of the reduction in activity observed at the 164 position. Additionally, as evident in Table 6 and Appendix 1, tryptophan differs greatly from lysine regarding hydrophobicity and volume. The insertion of a hydrophobic residue in place of a hydrophilic residue often leads to the destabilization of the local environment within the protein. However, this does not appear to occur in FNR at position 163. This suggests that the volume of the amino acid at 163 is more important to dimerization than hydrophobicity. This notion is supported by the severe reduction in activity observed in a previous KA163 FNR mutant (unpublished data). In both the KW163 and KA163 mutants, the positive charge was removed. However, KA163 resulted in a large decrease in volume occupied by the residue at 163. Further investigations into the role of K163 could begin with substitutions with the hydrophobic amino acid leucine or methionine. Leucine and methionine have neutral side chains that

occupy the same amount of volume as that of lysine (see Table 6) and could function to facilitate the same steric interactions as lysine. An insertion of a leucine/methionine residue in this position may yield further information regarding the importance of volume vs. hydropathy in stabilizing this hinge region in FNR. WT activity in the leucine or methionine mutant would suggest that the hydropathy of the residue at 163 is irrelevant.

LW146, MW147, MW157, and KW164 should be assayed in an ndh-lacZ strain.

FNR acts as a repressor at the *ndh* promoter. Thus, no activity in an *ndh-lacZ* strain would mean that FNR is dimerizing and binding to the *ndh* promoter to block the transcription of *lacZ*. LW146, MW147, MW157, and KW164 should be tested in a *ndh-lacZ* strain to ascertain whether or not the reduced activities observed in RZ8480 and RZ3293 were due to defects in dimerization or improper interactions with RNAP. To date, positions 146, 147, 157, and 163 have not been shown to lie along any of the proposed activating regions of FNR. This, however, does not exclude the possibility that they somehow influence the conformation of an activating region. Because both *narG* and *dmsA* are both class II promoters, it seems most likely that positions 146, 147, 157, and 163 would either affect ARI or ARIII with ARIII being the most likely candidate as it is dominant at class II promoters (5).

The fluorescence spectra of wild type FNR shows little contribution of tyrosine the fluorescence of FNR.

The fluorescence spectra of wild type FNR showed little intensity and little if any tyrosine fluorescence. We would expect a solvent exposed tyrosine to fluoresce near 303

nm. A small intensity was observed near this region at both 280 nm and 295 nm with the value at 295 nm being slightly less as expected based on tyrosine's maximal absorption being at 280 nm. Because the intensity in this region was small it is highly unlikely that it would mask any tryptophan fluorescence. In addition to this, tryptophan will not likely fluoresce in that same region. Therefore, if tyrosine is responsible for the fluorescent intensity observed near 305 nm, it is unlikely to be problematic in future tryptophan studies. The emission maxima observed in the wild type spectra $\varepsilon_{ex.}$ 280 and $\varepsilon_{ex.}$ 295 at 335 nm and 345 nm respectively are indicative of that obtained from tryptophan fluorescence. However, there are no tryptophan residues in the wild type protein. This suggests that this peak may be due to another protein containing Trp that co-purified with FNR. The wild type protein should be repurified and another set of anaerobic and aerobic spectra should be obtained to confirm the above analysis regarding the mimimal contribution of tyrosine to FNR fluorescence. After careful repurification, a protein gel should be run to check the purity of the sample. Because these initial spectra suggest there may be impurities in the protein sample, all future samples should be also be analyzed on a gel prior to fluorescence studies.

LH28 should be repurified and another set of spectra should be obtained.

The high intensity of fluorescence observed in LH28 at 345 nm is characteristic of a surface exposed tryptophan residue. This situation is similar to that observed in the wild type at an excitation of 295 nm. However, this is much more significant because the intensity is much greater. Clearly, there is a large contributor to fluorescence in the LH28 sample. Like the wild type FNR, LH28 contains no tryptophan. Therefore, the

observed signal is not coming from LH28. Due to the large nature of this signal it can be inferred that an impurity is present in large enough concentrations to make a significant contribution to the spectra obtained from LH28 under both anaerobic and aerobic conditions. Consequently, the notion that the spectra obtained is actually that of LH28 is questionable. LH28 could be run out on a gel with WT to check for impurities, but no more sample of either exists. Consequently, LH28 should be repurified and another set of spectra should be obtained.

Conclusion

In summary, these findings suggest that tryptophan insertions along the dimerization helix in positions 146, 147, and 157 and in the hinge region at position 164 may result in the inability of FNR to dimerize. This hypothesis will have to be tested further. However, a tryptophan insertion at position 163 does not appear to significantly inhibit dimerization. Purification of KW163 and subsequent fluorescence spectra under anaerobic and aerobic conditions could give useful information regarding the dimerization mechanism of FNR.

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