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A Region at the C-Terminus of the *Escherichia coli* Global Transcription Factor FNR Negatively Mediates Its Degradation by the ClpXP Protease

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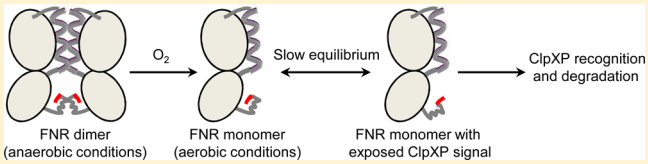
Supporting Information

ABSTRACT: The anaerobic global regulator FNR from *Escherichia coli* is a $[4Fe\text{-}4S]^{2+}$ cluster-containing dimer that is inactivated by O_2 through disruption of the Fe−S cluster and conversion to the monomeric apoprotein. It was shown that apo-FNR is subject to ClpXP proteolysis, and two recognition sites, amino acids 5−11 and amino acids 249 and 250, are responsible for targeting FNR to the protease. However, how the exposure of these sites is mediated such that only apo-FNR is recognized by the ClpXP protease and is degraded in a regulated manner so that a sufficient and similar FNR level is maintained in both anaerobic and aerobic conditions is unknown. To investigate this, we performed three-alanine scanning on amino acids 2−19 and 236−250 that are in the proximity of the two ClpXP recognition sites, and their functions remain unknown. We found that three-alanine substitution of residues 239−241 (LAQ239−241A_3) and 242−244 (LAG242−244A_3) caused reduced FNR protein levels, transcription activities, and growth rates under anaerobic conditions. In vivo degradation assays demonstrated that these mutants were degraded significantly faster than the wild type (WT), and either deletion of *clpXP* or blocking the ClpXP recognition site of amino acids 249 and 250 stabilizes these proteins. Circular dichroism analysis revealed that introduction of LAQ239−241A_3 caused conformational changes with a significant loss of secondary structures in both WT and an O_2 stable FNR dimer, FNR D154A. We propose that the region of amino acids 239−244 plays a negative role in the proteolysis of FNR by promoting a structural fold that limits the exposure of the proximal ClpXP site to the protease.



A facultative anaerobe such as *Escherichia coli* is able to switch between the aerobic and anaerobic modes of metabolism in response to the availability of O_2 in its environment. This metabolic switch is primarily controlled by a global transcription regulator called FNR (fumarate nitrate reduction).[1] Upon sensing O_2 deprivation, FNR activates or represses the expression of more than 100 genes in the *E. coli* genome,[2−4] allowing the bacterium to conserve energy by anaerobic respiration or fermentation when O_2, the favorable electron acceptor of the cellular respiration, is absent. To achieve a rapid and efficient transition between the aerobic and anaerobic lifestyle in the bacterium, both the intracellular FNR protein levels and its activities are exquisitely regulated.

The key property that allows FNR to act as an O_2 responsive transcription factor is its ability to dimerize upon binding of an O_2 labile $[4Fe\text{-}4S]^{2+}$ cluster, which is assembled during the de novo biosynthesis of FNR protein under anaerobic conditions. The dimerized FNR is then active in specific DNA binding and transcription regulation.[5−7] Upon exposure to O_2, the $[4Fe\text{-}4S]^{2+}$ cluster is rapidly oxidized to the $[2Fe\text{-}2S]^{2+}$ cluster, resulting in dissociation of the FNR dimer and the loss of transcription regulation. The $[2Fe\text{-}2S]^{2+}$ cluster is further destroyed by cellular superoxide species generated during aerobic metabolism, resulting in clusterless apo-FNR, which is the major form of the FNR protein present in aerobically grown *E. coli* cells.[8]

Although FNR exists as its apo form and is not active under aerobic conditions, it is present at a sufficient level similar to that of the FNR protein under anaerobic conditions.[8] It has been shown that maintaining a proper and similar FNR protein concentration under both conditions is essential for the proper physiological adaptation of *E. coli*.[9] Under anaerobic conditions, the optimal FNR protein level is primarily modulated at the transcriptional level through a negative feedback loop exploited by the $[4Fe\text{-}4S]^{2+}$ cluster containing FNR,[10] which binds to its own promoter and represses its expression. Under aerobic conditions, however, when FNR exists as its inactive form, the FNR protein level is known to be largely modulated at the post-translational level through ClpXP-mediated protein degradation.[9,11] It is reported that upon loss of the Fe−S cluster and dissociation of the FNR dimer, N-terminal amino acids 5−11 and C-terminal amino acids 249 and 250 in apo-FNR serve as two ClpXP recognition sites and target the protein to protease degradation.[9]

However, how the exposure of either or both of the ClpXP recognition sites is mediated in the different forms of FNR such that only the clusterless apo-FNR is recognized and degraded by ClpXP has not been resolved. Furthermore, it was

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demonstrated that apo-FNR is degraded at an unusually slow rate under aerobic conditions with an estimated half-life of 115 min in vivo.[9] How the degradation of apo-FNR is regulated in vivo is also unknown. Regulated proteolysis of transcription factors such as heat shock response regulator σ^{H} [12] and general stress response regulator σ^{S} [13] has been reported. However, the regulation is dependent on the levels or the status of their corresponding adaptor proteins, such as the availability of the chaperone proteins DnaK/J/GrpE for the degradation of σ^{H} and phosphorylation of the RssB protein for the degradation of σ^{S}. No adaptor protein was found to be required for the degradation of FNR, because in vitro proteolysis of the protein is even faster than that in vivo.[9] This suggests the presence of an intrinsic determinant for the regulated proteolysis of FNR.

In this study, we investigate this mechanism by performing three-alanine scanning on two regions of FNR: N-terminal amino acids 2−19 and C-terminal amino acids 236−250. These two regions are in the proximity of the two ClpXP recognition sites, yet their contribution to the structure and/or function of FNR remains obscure because of the lack of X-ray crystal structures of any forms of FNR and the absence of the corresponding regions in the prototype FNR/CRP superfamily protein cAMP receptor protein (CRP) (Figure S1 of the Supporting Information). Through three-alanine scanning, a region encompassed by amino acids 239−244 was shown to mediate the accessibility of the FNR protein by the ClpXP protease. This region is immediately upstream of the C-terminal ClpXP recognition site and is exclusively conserved in a subset of FNR orthologs that utilize $[4Fe\text{-}4S]^{2+}$ cluster-mediated dimerization as an O_2 sensing mechanism. We propose that FNR utilizes a region proximal to its C-terminal ClpXP recognition signal to mediate its accessibility by the ClpXP protease.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α and PK22,[6] which is a derivative of the BL21(DE3) strain, were utilized for cloning and overexpression of FNR proteins, respectively. A series of *E. coli* K-12 strains that lack the chromosomal *fnr* allele and contain a promoter−*lacZ* fusion of FNR-dependent genes, such as P_{narG}−*lacZ* (RZ8480) and P_{dmsA}−*lacZ* (PK3293),[6,15,16] were utilized as host strains for the β-galactosidase activity assay. These strains were transformed with pET11a-derived plasmids containing WT or various *fnr* mutants and were assayed for β-galactosidase activities to examine the effect of mutations on the transcriptional activity of FNR.

E. coli strains were cultured in LB broth or M9 glucose minimal medium as indicated in each of the experiments. Antibiotic concentrations in growth media were as follows: 100 μg/mL ampicillin, 20 μg/mL kanamycin, or 25 μg/mL chloramphenicol.

Construction of Strains and Plasmids. *Strains.* Strains AY0371 (RZ8480 *clpP*::*cat*), AY0372 (PK3293 *clpP*::*cat*), AY0373 (PK3293 *clpX*::*kan*), and AY0604 (PK3293 *clpP*::*cat clpX*::*kan*) were constructed by transduction of the alleles of *clpP*::*cat* (SG22508) and *clpX*::*kan* (SG22177)[9] to RZ8480 or PK3293 via P1*vir* transduction. Donor strains SG22508 and SG22177 were grown in LB and infected with P1*vir* in the presence of 10 mM $CaCl_2$ for 3 h. Subsequently, 200 μL of the freshly inoculated recipient strain (A_{600} ∼ 0.7) was incubated with 50 μL of the P1*vir*-infected donor strain for 30 min at 37 °C, followed by addition of 100 μL of 1 M citrate to terminate the infection. Transductants carrying the desirable allele with an antibiotic marker were screened on an LB plate supplemented with 4 mM citrate and appropriate antibiotics. The constructs were verified by colony polymerase chain reaction (PCR) and DNA sequencing.

Table 1. Bacterial Strains and Plasmids Used in This Study

construct	relevant genotype	ref or source
	E. coli Strains	
DH5α	F⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44 λ-thi-1 gyrA96 relA1*	our lab
PK22	BL21(DE3) Δ*crp*-bs*990 rpsL* Δ*fnr*ΩSp^r/Sm^r *zcj-3061*::Tn*10*	6
RZ8480	MG1655 *lacZ*Δ*145* Δ*fnr*ΩSp^r/Sm^r *narG*::MudI1734	6
PK3293	MG1655 *lacZ*Δ*145* Δ*fnr*ΩSp^r/Sm^r *λ*PC25	16
SG22508	MC4100 *clpP*::*cat*	9
SG22177	MC4100 *clpX*::*kan*	9
AY0371	RZ8480 *clpP*::*cat*	this study
AY0372	PK3293 *clpP*::*cat*	this study
AY0373	PK3293 *clpX*::*kan*	this study
AY0604	PK3293 *clpX*::*kan clpP*::*cat*	this study
	Plasmids	
pET11a	Ap^r, phage T7 gene 10 promoter	Novagen
pET28a	Kn^r, phage T7 promoter, His tag	Novagen
pACYC184	*Cm*^{r}	NEB
pPK823	Ap^r, *fnr* +1 to +1115 in pET11a *Nde*I/*Bam*HI	6
pAY0934	Kn^r, *fnr* +1 to +1115 in pET28a *Nde*I/*Bam*HI	this study
pRZ7411	*Cm*^{r}, *fnr* −521 to +1115 in pACYC184 *Hin*dIII/*Bam*HI	6

Plasmids. Three-alanine (3-Ala) block substitutions were constructed by site-directed mutagenesis. Amino acid residues 2−19 and 236−250 of FNR were successively replaced with three alanines, while native alanine residues remained unchanged. A diagrammatic representation of these mutations is summarized in Figure 1. To construct 3-Ala substitution mutants, a pair of complementary primers comprising 45−50 nucleotides with desired mutation sites located in the center were synthesized upon request (Invitrogen). Site-directed mutagenesis PCR was conducted using pPK823 (pET11a-*fnr*)[6] or pRZ7411 (pACYC184-*fnr*)[6] as a template in the presence of high-fidelity DNA polymerase *PfuTurbo* (Stratagene) or *iProof* (Bio-Rad). The PCR product was subsequently digested with *Dpn*I (NEB) to remove the remaining template plasmid and was transformed into *E. coli* strain DH5α. Selected single colonies were subjected to propagation in LB broth supplemented with the proper antibiotics. Plasmids were prepared and verified by DNA sequencing. The same approach was utilized to construct single-amino acid substitution and deletion mutants utilized in this study. The three-alanine substitution mutants are designated with the native amino acids followed by the position and A_3; i.e., LAQ239−241A_3 indicates the L, A, and Q amino acids at positions 239−241, respectively, were replaced with three alanine residues.

The verified plasmids containing the WT or mutant *fnr* allele in pET11a or pACYC184 were transformed into RZ8480 or PK3293 for the β-galactosidase activity assay. Hexa-His-tagged *fnr*, *fnr* D154A, *fnr* LAQ239−241A_3, and *fnr* D154A LAQ239−241A_3 were constructed in a pET28a vector and

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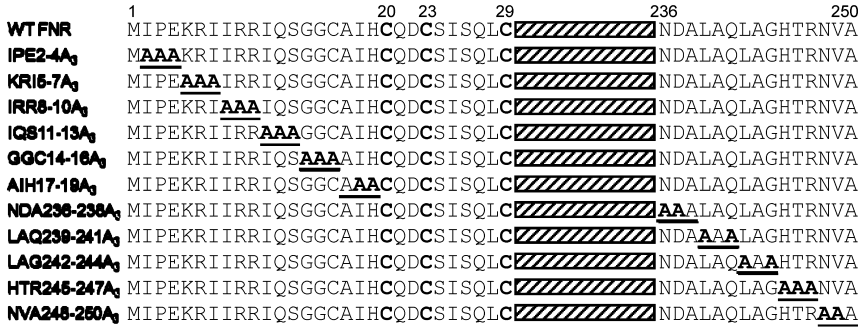

Figure 1. Diagram of 3-Ala block substitution mutants in the N- and C-terminal region of FNR. Amino acids 2−19 and 236−250 were successively replaced with three alanines (underlined and bold), while the native alanine residues remained unchanged. Three of four essential cysteines in the N-terminal region are shown in bold. Three-alanine substitution mutants are designated with the native amino acids followed by the position and A_3; i.e., LAQ239−241A_3 indicates amino acids L, A, and Q at positions 239−241, respectively, are replaced with three alanine residues.

transformed into strain PK22 for IPTG induction and protein purification.

Transcription Activity Assay. In vivo transcriptional activities of FNR and its variants were determined by a β-galactosidase assay[17] using *E. coli* strain RZ8480 or PK3293 transformed with the corresponding plasmid. *E. coli* cells were inoculated (final cell density of ~10^3 cells/mL) in screw-capped Pyrex culture tubes filled with M9 glucose minimal medium supplemented with nutrients {0.2% glucose, 0.2% casamino acids, 4 μg/mL thiamine, 100 μM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/mL ferric ammonium citrate, 0.2 μM ammonium molybdate, 1.4 mM KNO_3 [for RZ8480 (P_{narG}−*lacZ*) only], and appropriate antibiotics} and grown without being shaken at 37 °C. Anaerobiosis of *E. coli* grown in this device is achieved with the following factors: (i) a very small amount of initial inoculum (~10^3 cells/mL), which will rapidly consume the residual O_2 dissolved in the growth medium, resulting in an O_2 free environment in the culture tube; (ii) the use of a screw-capped Pyrex culture tube with an O_2 indiffusible cap filled with growth medium; and (iii) no aeration. After A_{600} had reached 0.3, chloramphenicol (50 μg/mL) or tetracycline (20 μg/mL) was added to terminate cell growth and protein synthesis, and cultures were placed on ice until they were assayed. The assay was performed as described by Mettert et al.[10] Results were obtained from three independent isolates and presented as the mean percentage relative to that of WT FNR.

Western Blot. Cells grown under the same conditions that were used for β-galactosidase activity assays were also subjected to Western blot analysis. The amount of cells for the Western blot was normalized to be equivalent to 300 μL (for cells transformed with the pET11a-derived plasmids) or 200 μL (for cells transformed with the pACYC184-derived plasmids) of cells with an A_{600} of ~0.3. Cell pellets were resuspended in 15 μL of SDS sample buffer followed by boiling to denature the proteins. The resulting suspension was centrifuged for 2 min at 13000 rpm, and the supernatant was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Proteins separated via SDS−PAGE were electrically transferred to a nitrocellulose membrane (Bio-Rad) for 90 min and subsequently blocked with 5% nonfat milk in TBST for 1 h at room temperature. Following incubation with the polyclonal anti-FNR antibody (a gift from P. Kiley, University of Wisconsin, Madison, WI) and subsequent blotting with the goat anti-rabbit IgG HRP-conjugated secondary antibody (Bio-Rad) for 2 h, images of FNR proteins were obtained by X-ray film development (Medical X-ray Processor, Kodak, Rochester, NY) following treatment with the ECL Plus Western Blotting Detection Reagents (GE Healthcare). Signals corresponding to FNR proteins were quantified with ImageJ (National Institutes of Health) and are presented as percentages relative to the level of WT FNR, which was treated and examined under the same conditions. Results are means from at least two independent isolates.

Growth Curve. RZ8480 cells harboring pET11a-*fnr* as well as the mutant variants were cultured in M9 minimal medium supplemented with nutrients as described above, except 0.2% glucose was replaced with 0.4% glycerol and nitrate (1.4 mM KNO_3) was added as the terminal electron acceptor. Cells were grown under anaerobic conditions as described above, and the absorbance at 600 nm was measured (SPECTRONIC 20D+, Thermo Scientific) every 1 h. The growth curve was plotted using A_{600} values on the *Y*-axis and time of growth on the *X*-axis. Values at each of the time points were means from two independent isolates.

In Vivo Degradation Analysis. RZ8480 cells transformed with pPK823 (pET11a-*fnr*), pRZ7411 (pACYC184-*fnr*), or the various 3-Ala substitution mutants were grown aerobically in M9 glucose minimal medium supplemented with nutrients at 37 °C and 220 rpm until the cells reached the early exponential phase (A_{600} ~ 0.1). Cell growth and protein synthesis were terminated by adding chloramphenicol (50 μg/mL) or tetracycline (20 μg/mL), and the time was designated as time zero. Cultures were continuously shaken at 37 °C. Equal amounts of cells were removed from the cell culture after 60, 120, 180, and 240 min (for pACYC184-*fnr*-LAQ239−241A_3, time points of 20, 40, 60, and 80 min were used). Cell pellets were subjected to Western blot analysis as mentioned above to quantify FNR protein at each of these time points. FNR protein levels are presented as percentages relative to that at time zero, and the half-life ($t_{1/2}$) was the mean value of $t_{1/2}$ calculated from equation $t_{1/2} = t/\log_2[N_0/N(t)]$, where N_0 is the initial FNR protein level and t represents time. The in vivo degradation curve of each of the constructs was plotted using the fitted equation $y = N_0 \times 2^{-x/t_{1/2}}$ with the corresponding $t_{1/2}$ value.

Purification of His-Tagged WT and Mutant FNR Proteins. PK22 transformed with pET28a-his$_6$-*fnr* or mutants was grown in LB supplemented with 0.2% glucose overnight. After 1:100 dilution into 100 mL of LB, cells were grown aerobically to an A_{600} of ~0.6 followed by induction with 0.4 mM IPTG for 4 h at 30 °C. Cells were harvested by centrifugation at 6000 rpm for 10 min, and cell pellets were collected and resuspended in 4 mL of lysis buffer containing 50

mM KPO_4 (pH 6.8), 10% glycerol, 150 mM NaCl, 0.5 mM DTT, 0.15 mM PMSF, and 0.25 mg/mL lysozyme. To lyse the cells, the cell suspension was placed on ice and subjected to sonication (SONIFIER 450, VMR Scientific) for 10 s followed by 10 s interval for 10 cycles (output of 10, duty cycle of 30%). The cell lysate was then ultracentrifuged (XL-90 Ultracentrifuge, BECKMAN) at 20000 rpm for 30 min at 4 °C. The supernatant was collected and incubated with 1 mL of Ni Sepharose 6 Fast Flow resin (GE Healthcare) at 4 °C for 1 h. The Sepharose/protein slurry was subsequently loaded into a gravity flow Econo-Column (Bio-Rad). Unbound and nonspecifically bound proteins were removed when the samples were washed with 10 bed volumes of phosphate buffer containing 40 mM imidazole (Sigma). His_6-tagged FNR or mutants were eluted with the same phosphate buffer containing 500 mM imidazole. The purity of the eluted protein was analyzed by SDS−PAGE and was >95%.

Circular Dichroism (CD) Measurement. Purified His_6-tagged FNR or mutants (5 μM) were subjected to dialysis using Spectra/Por membrane tubing (Spectrum, MW of 6000−8000) in 50 mM KPO_4 (pH 6.8) buffer with 20 mM K_2SO_4 at 4 °C overnight to remove imidazole and chloride ions. CD spectra of His_6-FNR and mutants were recorded on a J-815 CD spectropolarimeter (Jasco) equipped with a 1.0 mm path length quartz cell at room temperature. The samples were measured at a scanning speed of 100 nm/min from 300 to 190 nm with a 1.0 nm data pitch and a 1.0 nm bandwidth. Adaptive smoothing of raw data was achieved with Jasco Spectra Analysis (Means-Movement, convolution width of 5), and the secondary structure was predicted by the K2d algorithm[18] using the 200−240 nm data.

RESULTS

Three-Alanine Substitution Mutants of Amino Acids 2−19 and 236−250 of FNR Exhibit Altered Protein Levels and Transcription Activities under Anaerobic Conditions. To investigate the functions of amino acid residues 2−19 and 236−250 of FNR and their potential roles in mediating the proteolysis of FNR by the ClpXP protease, we constructed 3-Ala substitution mutants in these regions. The region of amino acids 20−29 was not selected because three of the four essential cysteine residues that ligate the Fe−S cluster into FNR are located in this region[19,20] and alanine substitution is likely to cause undesirable disruption of the Fe−S cluster binding as indicated by previous studies.[15,21] The wild type or various alanine substitution mutants were cloned into plasmid pET11a behind the T7 promoter and were transformed into strains containing chromosomal promoter−*lacZ* fusions of FNR-dependent genes: P_{narG}−*lacZ* (RZ8480) or P_{dmsA}−*lacZ* (PK3293) for the β-galactosidase activity assay, which serves as an indicator of the activity and FNR protein level in the cell. Cells for this assay were cultured without IPTG induction, as Moore et al.[22] had shown that FNR levels produced from this plasmid in the absence of T7 RNA polymerase did not exceed the single-copy level of FNR on the chromosome and were suitable for the study of the effect of various FNR mutants.

The β-galactosidase activity assay of P_{narG}−*lacZ* showed that substitution of most of the amino acids in these two regions with alanine did not have a significant effect on FNR transcriptional activity except for 3-Ala substitutions at positions 5−7 (KRI5−$7A_3$) and 242−244 (LAG242−$244A_3$) that displayed moderate decreases in activity and positions 17−19 (AIH17−$19A_3$) and 239−241 (LAQ239−$241A_3$) that displayed severe impairments compared with that of WT (Figure 2A). Similar results were obtained with the P_{dmsA}−*lacZ*

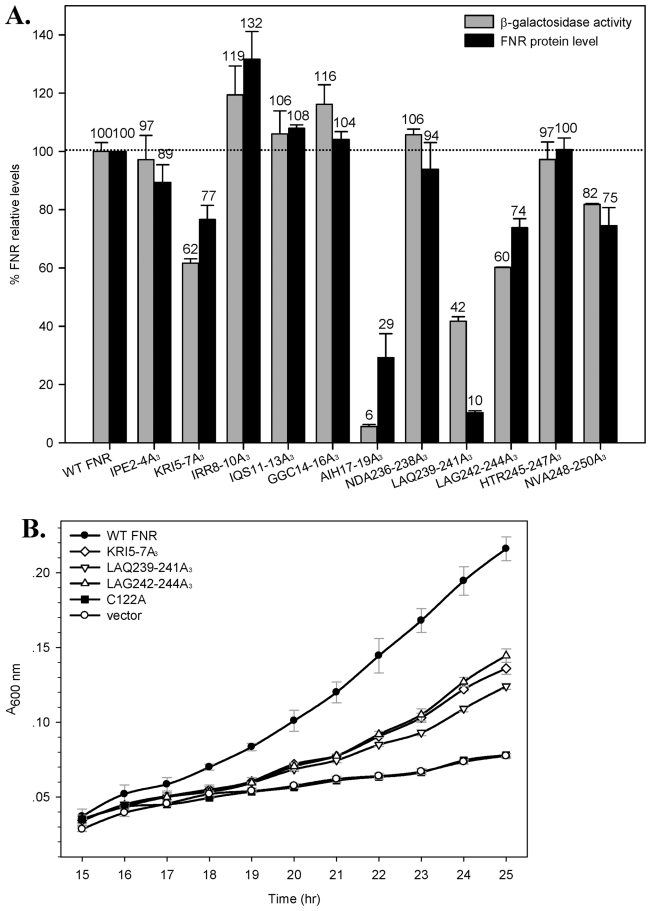

Figure 2. Effect of N- or C-terminal 3-Ala substitution on the transcriptional activity, FNR protein level, and growth rate of *E. coli* under anaerobic conditions. WT or FNR mutants were expressed from the pET11a vector without IPTG induction. (A) Transcription activities (gray) and FNR protein levels (black) of the mutants under anaerobic conditions. Transcription activities were determined by β-galactosidase activities from P_{narG}−*lacZ* that are activated by WT or various FNR 3-Ala block mutants as indicated, and FNR protein levels were determined by Western blot analysis using the anti-FNR antibody. Values are mean percentages relative to that of WT FNR. (B) Growth curves of *E. coli* strains carrying the pET11a vector, WT FNR, FNR C122A, and selective 3-Ala block mutants, KRI5−$7A_3$, LAQ239−$241A_3$, and LAG242−$244A_3$, in M9 minimal medium supplemented with glycerol (0.4%) and nitrate (1.4 mM) under anaerobic conditions. Error bars represent the deviation of A_{600} of two independent isolates.

(PK3293) reporter system, suggesting that the effect of these substitution mutants was not promoter specific. To investigate whether the decreased activities in selective mutants were due to the changes in FNR protein levels, we performed Western blot analysis and showed that protein levels of these mutants were reduced compared with the WT level (Figure 2A). Furthermore, protein levels in each of the mutants largely correlated with the levels of β-galactosidase activity, suggesting that 3-alanine substitution in these regions affects the cellular FNR activities through their effect on FNR protein levels. It is noteworthy that this observation is not common in previously characterized FNR mutants, such as those impaired in cluster assembly (C122A), DNA binding (E209K and S212R),

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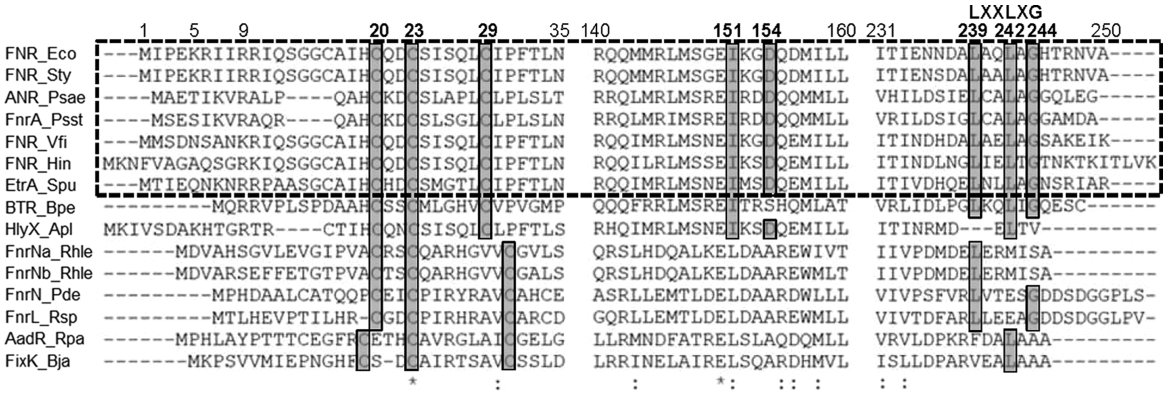

Figure 3. Sequence alignment of various FNR orthologs demonstrating the conservation of the stretch of $L^{239}XXL^{242}XG^{244}$. Sequence alignment of *E. coli* FNR and other 14 orthologs[27] was performed with UniProt (http://www.uniprot.org). Proteins, organisms, and entry numbers are given: FNR_Eco (*E. coli* K-12, P0A9E5), FNR_Sty (*Salmonella typhimurium*, P0A2T8), ANR_Psae (*Pseudomonas aeruginosa*, P23926), FnrA_Psst (*Pseudomonas stutzeri*, P47200), FNR_Vfi (*Vibrio fischeri*, Q70ET4), FNR_Hin (*Haemophilus influenzae*, P45199), EtrA_Spu (*Shewanella putrefaciens*, E6XLG6), BTR_Bpe (*Bordetella pertussis*, Q08530), HlyX_Apl (*Actinobacillus pleuropneumoniae*, P23619), FnrNa_Rhle (*Rhizobium leguminosarum*, P24290), FnrNb_Rhle (*R. leguminosarum*, Q52775), FnrN_Pde (*Paracoccus denitrificans*, Q51677), FnrL_Rsp (*Rhodobacter sphaeroides*, P51007), AadR_Rpa (*Rhodopseudomonas palustris*, Q01980), and FixK_Bja (*Bradyrhizobium japonicum*, P29286). Conserved residues are boxed in gray, and numbers are adjusted using FNR_Eco as the reference. Conservation of $L^{239}XXL^{242}XG^{244}$ (bold) as well as the correlation of their conservation with I151 and D154 (bold) in a subset of orthologs is highlighted in a dashed box. This subset of FNR orthologs utilize Cys-X_2-Cys-X_5-Cys (bold) to bind the $[4Fe\text{-}4S]^{2+}$ cluster.

interaction with RNAP (R189E), or dimerization (I151A) (Figure S2 of the Supporting Information). In those mutants, although FNR transcription activities were abolished or decreased significantly, they showed steady-state FNR protein levels similar to that of WT. This indicated that the correlation between the activities and FNR protein levels was specific to the regions of the N- and C-termini of FNR we examined.

To further confirm this, we examined the effect of selective 3-Ala substitution mutants on the growth of *E. coli* during anaerobic respiration, i.e., cells grown anaerobically using glycerol as the carbon source and nitrate as the terminal electron acceptor. RZ8480 transformed with the WT, pET11a vector, or FNR C122A, which abolishes $[4Fe\text{-}4S]^{2+}$ cluster binding and FNR activity under anaerobic conditions as shown previously,[23] were utilized as controls. As expected, cells in which the *fnr* allele was deleted but transformed with the vector control or FNR C122A showed almost no growth compared with that transformed with pET11a-*fnr* during anaerobic respiration. The growth of alanine substitution mutants KRI5−7A_3, LAQ239−241A_3, and LAG242−244A_3 was slower than that of WT but faster than that of the negative controls (Figure 2B), consistent with the decreased intracellular FNR protein levels in these mutants compared with the WT level. Because the low activity of AIH17−19A_3 has been addressed previously and was attributed to disruption of the Fe−S cluster binding of FNR because of their proximity to the essential Cys20,[21] we focused on other alanine substitution mutants, particularly KRI5−7A_3, LAQ239−241A_3, and LAG242−244A_3.

Three-Alanine Substitutions Did Not Disrupt Fe−S Cluster Binding. Because $[4Fe\text{-}4S]^{2+}$ cluster-induced FNR dimerization is essential to prevent the proteolysis of FNR protein, we first asked whether those 3-Ala block substitutions that showed reduced FNR protein levels were due to the loss of the $[4Fe\text{-}4S]^{2+}$ cluster. To test this, we introduced the C122A substitution, which has been shown to eliminate Fe−S cluster binding upon introduction into WT FNR and subsequently abolish its activity,[20,23] into the 3-Ala block substitution mutants. It was shown that after incorporation of the C122A substitution, activities of these mutants were completely abolished irrespective of the variable FNR protein levels in these mutants (data not shown), suggesting that the activities of the 3-Ala block substitutions were still dependent on the Fe−S cluster. Furthermore, comparing the activities of these mutants under anaerobic conditions, in the range of 120−240 Miller units, with those under aerobic conditions, <10 Miller units, also suggests that these alanine substitution mutants maintained the ability to bind Fe−S clusters and to sense and respond to O_2.

To explore how then these amino acids affect FNR protein levels in *E. coli*, we first performed sequence alignment and examined the conservation or similarity of these amino acid residues among the various FNR orthologs. We found that a stretch of "LXXLXG" encompassed by L239, L242, and G244 in *E. coli* is present and exclusively conserved among a subset of FNR orthologs that contain the Cys-X_2-Cys-X_5-Cys signature at their N-termini to bind the $[4Fe\text{-}4S]^{2+}$ cluster but is absent in those orthologs containing the Cys-X_2-Cys-X_7-Cys signature such as FnrN and FnrL, or those that lack the N-terminal Cys ligands such as FixK (Figure 3).[24] Furthermore, the conservation of this stretch is also largely correlated with that of I151 and D154, two signature amino acids in the dimerization helix of those FNR orthologs that utilize the $[4Fe\text{-}4S]^{2+}$ cluster-mediated dimerization mechanism to sense O_2. These information implies the functional significance of amino acids 239−244 in FNR orthologs. On the other hand, although substitution of amino acids 5−7 with alanine also caused reduced transcription activities and FNR protein levels in our assay, they were not conserved among the various FNR orthologs; thus, we next focus on three-alanine substitution mutants LAQ239−241A_3 and LAG242−244A_3.

Alanine Substitutions of Amino Acids 239−241 and 242−244 Accelerate FNR Protein Degradation. Because introduction of alanine substitutions at positions 239−241 and 242−244 caused decreased FNR protein levels, we next examined whether this is due to accelerated degradation of these proteins. To test this, we performed an in vivo degradation assay under aerobic conditions because (i) under aerobic conditions FNR exists as its clusterless, monomeric

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form, thus excluding the effect of the loss of the Fe−S cluster on the acceleration of protein degradation, and (ii) introduction of these alanine substitutions had an effect on FNR protein levels that was of the same magnitude under both aerobic (data not shown) and anaerobic conditions. Another mutant, HTR245−247A$_3$, whose steady-state protein level was not decreased in our analysis shown above, was selected as a control. As shown in Figure 4, degradation of LAQ239−241A$_3$

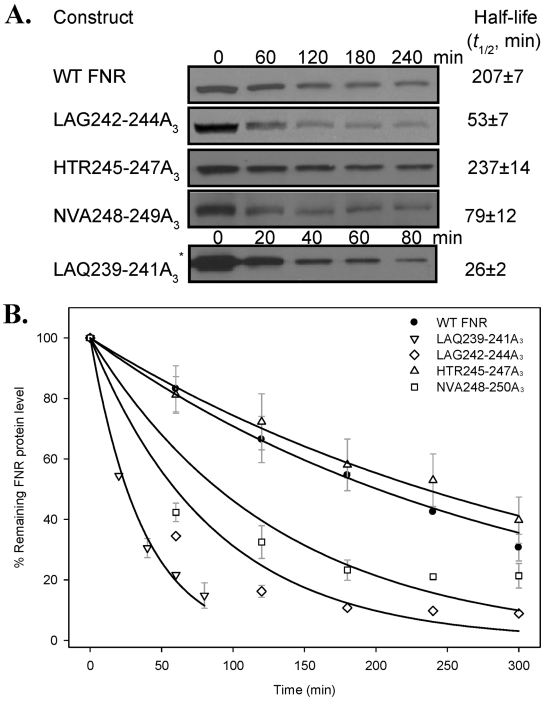

Figure 4. In vivo degradation of WT and selective 3-Ala block substitution variants. (A) Western blot analysis of FNR protein levels at the various time points following termination of protein synthesis. WT and mutants LAG242−244A$_3$, HTR245−247A$_3$, and NVA248−250A$_3$ were expressed from the pET11a vector without IPTG induction. Mutant LAQ249−241A$_3$ was expressed from the pACYC184 vector to achieve a fairly detectable level of FNR protein. The half-life of each of the constructs was calculated by the equation $t_{1/2} = t/\log_2[N_0/N(t)]$ as described in Materials and Methods. (B) In vivo degradation curves of WT and selective 3-Ala substitution mutants. Percentages of remaining FNR protein levels relative to that of time zero for each of the constructs are shown on the *Y*-axis. The curve was derived from the equation $y = N_0 \times 2^{-x/t_{1/2}}$ using calculated $t_{1/2}$ for each of the constructs.

and LAG242−244A$_3$ was faster than that of the WT or HTR245−247A$_3$. It is noteworthy that mutant LAQ239−241A$_3$ ($t_{1/2} \sim 26$ min) was degraded significantly faster than the WT ($t_{1/2} \sim 200$ min) and other mutants ($t_{1/2} = 50$−80 min). In fact, because of the exceptionally rapid degradation of LAQ239−241A$_3$, which led to a dramatically low level of FNR protein in the cell, the in vivo degradation assay of this mutant was examined in the construct in which FNR is expressed from a low-mid-copy plasmid pACYC184 to achieve a fairly detectable initial FNR protein level. This result demonstrated that alanine substitution of amino acids 239−241 and 242−244 accelerated the degradation of FNR protein.

To exclude the possibility that the accelerated degradation of these alanine substitution mutants was due to the artificial introduction of a ClpXP recognition site, we constructed mutants in which L239, L239 and A240, or L242 and A243 were replaced with one or two aspartic acids, because previous studies had demonstrated that small hydrophobic amino acid residues such as alanine favor ClpXP binding and replacing these residues with aspartic acids can prevent the recognition and binding by ClpXP.[25] Analysis of β-galactosidase activity and protein levels of these mutants demonstrated that introduction of aspartic acid at these positions, L239D, LA239−240DD, and LA242−243DD mutants, did not result in the restoration of protein levels or activities of the mutations (Figure 5). In

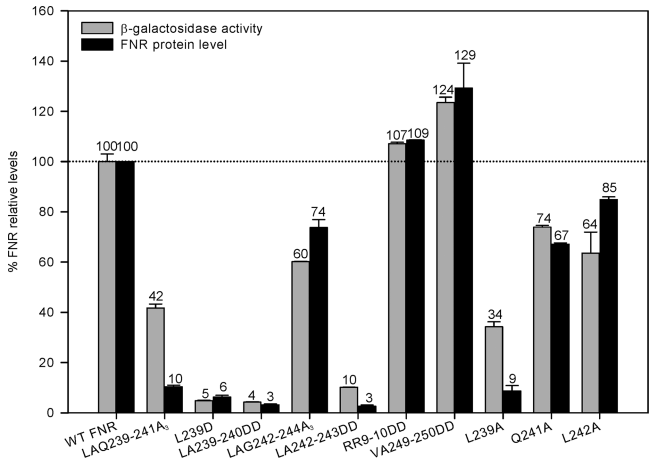

Figure 5. Defect of 3-Ala substitution mutants LAQ239−241A$_3$ and LAG242−244A$_3$ is not due to the introduction of artificial ClpXP recognition sites. L239A and L242A are responsible for the defects in their respective 3-Ala substitution mutants. β-Galactosidase activities and FNR protein levels of FNR variants in which amino acid(s) L239, LA239−240, LA242−243, and RR9−10 and VA249−250 in the two existing ClpXP sites were replaced with aspartic acids (D) to examine whether the 3-Ala substitution created artificial ClpXP sites. In addition, amino acid L239, Q241, or L242 was replaced with alanine (A) to examine the roles of individual amino acids in LAQ239−241A$_3$ and LAG242−244A$_3$. The β-galactosidase activity assay and Western blot analysis for measuring FNR protein levels are described in Materials and Methods.

contrast, substituting the amino acids of the two existing ClpXP binding sites of FNR, RR9−10 and VA249−250, with aspartic acids resulted in FNR protein levels greater than that of the WT, indicating the effectiveness of introducing aspartic acids at the ClpXP recognition site in preventing ClpXP-dependent proteolysis (Figure 5). These results suggested that decreased FNR protein levels in LAQ239−241A$_3$ and LAG242−244A$_3$ were not due to the introduction of an artificial ClpXP recognition site. To test whether substitution of LAQ239−241 and LAG242−244 with alanine created an efficient recognition site for other proteases, such as ClpAP, Lon, and HslUV,[26] we tested LAQ239−241A$_3$ and LAG242−244A$_3$ protein levels in strains that included deletions of either individual or different combinations of these proteases[27] and found that inactivation of none of these proteases could restore FNR protein levels (data not shown). These results together suggest that amino acids 239−244 intrinsically mediate the proteolysis of FNR protein in a negative manner and alanine substitution of these amino acids likely removed this negative effect, causing accelerated FNR degradation.

To confirm this notion, we blocked the C-terminal ClpXP recognition site in these two mutants, i.e., replacing VA249−250 with DD in LAQ239−241A$_3$ and LAG242−244A$_3$, and examined whether disrupting the ClpXP recognition site would

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restore their protein levels. As expected, incorporation of VA249−250DD significantly restored FNR protein levels of LAQ239−241A$_3$ and LAG242−244A$_3$. Likewise, blocking the two ClpXP recognition sites in FNR via introduction of RR9−10DD and VA249−250DD caused a significant increase in FNR protein level compared with that of the WT (Figure 6A). The restoration is also confirmed by the growth curve assay that demonstrated that blocking the C-terminal ClpXP site can promote the growth of LAQ239−241A$_3$ and LAG242−244A$_3$ under the anaerobic respiratory conditions (Figure 6B). Furthermore, in vivo degradation experiments demonstrated that LAQ239−241A$_3$ and LAG242−244A$_3$ were stabilized in the Δ*clpXP* strain (Figure 6C), confirming the negative role of amino acids 239−244 in the regulated proteolysis of FNR, and 3-Ala substitution of these amino acids led to accelerated degradation of FNR by ClpXP. We also tried to examine the steady-state FNR protein levels of these two mutants in the Δ*clpXP* strain but failed to observe either restoration of the mutant proteins or an elevated WT protein level versus that in ClpXP$^+$ strains. This may be attributed to the pleiotropic effects of the Δ*clpXP* strain in M9 glucose medium, which showed slower growth than the WT, a defect in cell division, and a reduced level of FNR synthesis as reported by Mettert et al.[9]

L239 and L242 Are Primarily Responsible for the Defects in LAQ239−241A$_3$ and LAG242−244A$_3$, Respectively. To dissect the contribution of each of the amino acids in the region of amino acids 239−244, we mutated individual amino acid residues to alanine, i.e., L239A, Q241A, and L242A, and examined the effect of these individual mutants on FNR activities and protein levels. Gly244 was not selected for mutation to alanine, as the properties of these two amino acids are very similar. It was shown that L239 and L242 were largely responsible for the significant decrease in the activity and FNR protein level observed in the 3-Ala substitution mutants LAQ239−241A$_3$ and LAG242−244A$_3$, respectively (Figure 5), suggesting that a hydrophobic long alkyl side chain is required at these two positions to negatively mediate the proteolysis of FNR protein by ClpXP.

Mutation of Amino Acids 239−241 Causes Conformational Changes in FNR. To investigate how these amino acids prevent the accessibility of FNR by the ClpXP protease, we first examined the possibility that amino acids 239−244 contribute to the dimerization of FNR protein given the conservation of the L^{239}XXL^{242}XG^{244} motif in those FNR orthologs that contain I151 and D154, two signature amino acids in the dimerization helix of the protein. Because mutation of amino acids 239−241 had the most significant effect on both the degradation of FNR and the steady-state FNR levels in the studies shown above, we focused on mutant LAQ239−241A$_3$ in the following in vitro studies. We used an O_2 stable FNR dimer, FNR D154A, and examined whether introduction of LAQ239−241A$_3$ reduces its level of dimerization. Gel filtration analysis revealed that both FNR D154A and FNR D154A LAQ239−241A$_3$ displayed ~80% dimer under aerobic conditions (at 20 μM), and introduction of the LAQ239−241A$_3$ substitution did not significantly alter the ratio of dimer (~80%) and monomer (~20%) of FNR D154A (at 20 μM) (data not shown). We then performed CD analysis of WT FNR and FNR LAQ239−241A$_3$, as well as O_2 stable dimer FNR D154A and its LAQ239−241A$_3$ derivative, to examine whether mutations of amino acids 239−241 cause conformational changes in FNR. CD spectra of these proteins revealed that introduction of the LAQ239−241A$_3$ substitution into WT FNR or FNR D154A

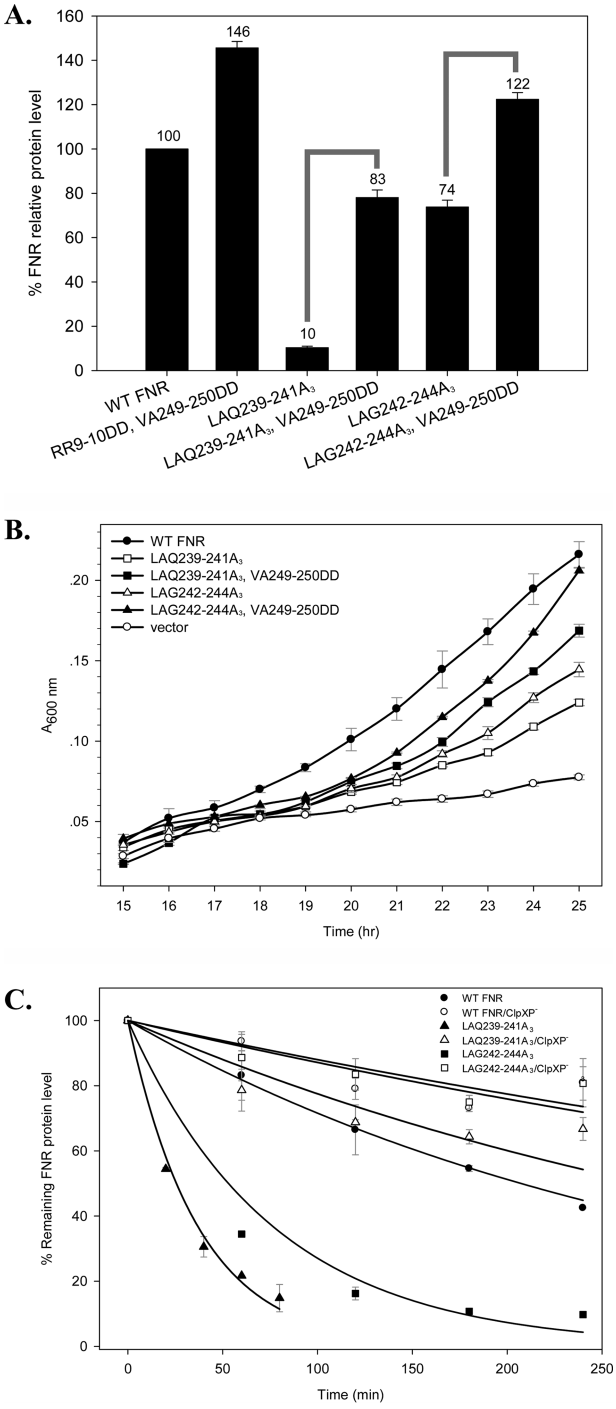

Figure 6. Stabilization of LAQ239−241A$_3$ and LAG242−244A$_3$ by introduction of VA249−250DD, which blocks ClpXP recognition and binding, or by deletion of *clpXP*. (A) FNR protein levels of WT, LAQ239−241A$_3$, and LAG242−244A$_3$ as well as their respective VA249−250DD incorporation variants. (B) Growth of these constructs in M9 minimal medium supplemented with glycerol (0.4%) and nitrate (1.4 mM) under anaerobic conditions. Introduction of VA249−250DD restored the poor growth of LAQ239−241A$_3$ and LAG242−244A$_3$. (C) In vivo degradation curves of the WT (●), LAQ239−241A$_3$ (▲), and LAG242−244A$_3$ (■) in the ClpXP$^+$ strain or the WT (○), LAQ239−241A$_3$ (△), and LAG242−244A$_3$ (□) in the ClpXP$^-$ strain. Deletion of *clpXP* (ClpXP$^-$) stabilizes these proteins.

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caused a significant loss of secondary structure in both proteins (Figure 7A,B), whereas FNR and FNR D154A exhibited very

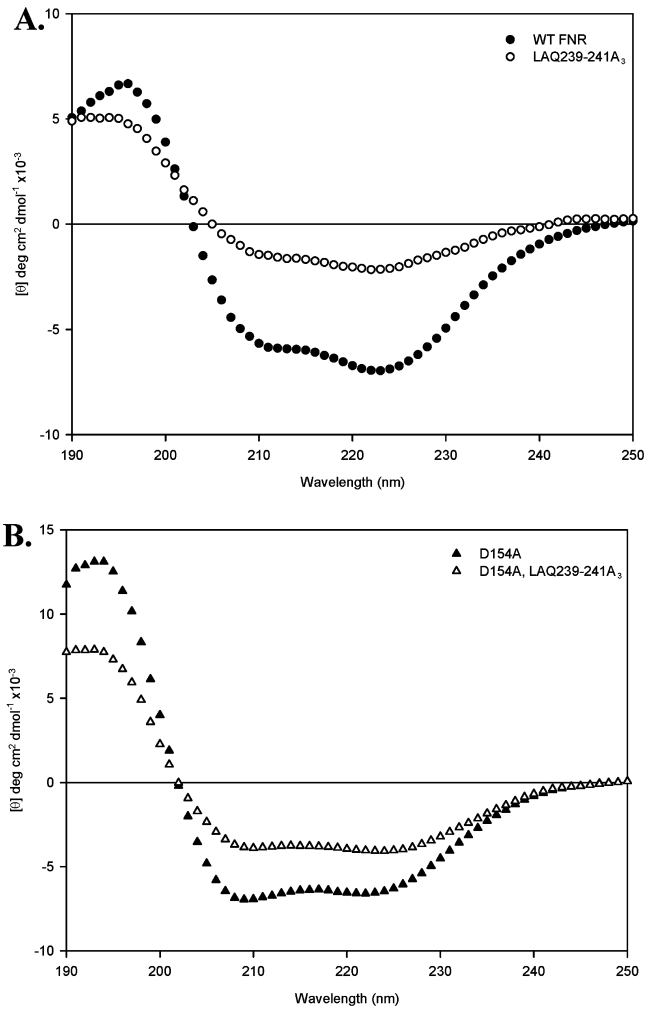

Figure 7. Mutation of amino acids 239−241 affects the conformational integrity of FNR as revealed by the CD spectra of WT FNR, FNR LAQ239−241A_3, FNR D154A, and FNR D154A LAQ239−241A_3. (A) Sectra of 5 μM His_6-FNR (●) and His_6-FNR LAQ239−241A_3 (○). Introduction of the LAQ239−241A_3 substitution caused the loss of secondary structures of FNR. (B) Spectra of 5 μM His_6-FNR D154A (▲) and His_6-FNR D154A LAQ239−241A_3 (△). Introduction of the LAQ239−241A_3 substitution caused a similar loss of certain secondary structures in FNR D154A.

similar spectra, consistent with previous studies that showed these two proteins displayed similar secondary structures.[6,14] This result suggests that amino acids 239−241 (perhaps amino acids 239−244 based on our in vivo data) play a role in maintaining the structural integrity of FNR protein presumably in the local environment of the C-terminal ClpXP site VA249−250 and conformational changes caused by mutation of these residues led to the exposure of this ClpXP recognition site. Because FNR D154A has been shown to display a secondary structure similar to that of the native FNR dimer,[6,14] the conformational changes following the introduction of the substitution of LAQ239−241 into FNR D154A suggest that alanine substitution of LAQ239−241 has a similar effect on the conformational integrity of the FNR dimer as that in the monomeric FNR, consistent with our observation that LAQ239−241 also caused decreased FNR protein levels under anaerobic conditions.

DISCUSSION

Unlike certain regulatory proteins in bacteria whose presence and levels are dependent on the availability of the corresponding inducing signals, FNR is present at a similar and sufficient amount in the presence and absence of its responsive signal O_2. Its activation during anaerobiosis is exclusively dependent on $[4Fe\text{-}4S]^{2+}$ cluster binding and consequent protein dimerization.[6] Because a higher intracellular FNR concentration can lead to nonspecific, Fe−S cluster-independent dimerization,[9] it is important to restrict the FNR protein level to a proper range especially under aerobic conditions, such that no unnecessary activation of FNR-dependent genes occurs under this condition. Thus, elucidating the intrinsic mechanisms in FNR that mediate the recognition and degradation of FNR by the ClpXP protease is essential to fully understanding the O_2 sensing mechanism of FNR. In this study, we demonstrated that a C-terminal region of FNR encompassed by amino acids 239−244 constitutes a determinant that mediates the accessibility of FNR protein by the ClpXP protease and thus contributes to an additional layer of regulation of the proper O_2 sensing capacity of FNR.

FNR was found to be a proteolytic substrate of ClpXP by both co-immunoprecipitation[11] and pulse-chase experiments.[9] It was found that both the N- and C-terminal recognition signals, amino acids 5−11 and amino acids 249 and 250, respectively, are required for ClpXP-mediated degradation.[9] However, how the exposure of either or both of these two recognition sites is modulated such that only the clusterless apo-FNR is targeted to proteolysis and is degraded at a rather slow rate is unknown. We contemplated that because ClpXP is a housekeeping protease in *E. coli*, one or both of the ClpXP recognition sites of FNR must be shielded in the $[4Fe\text{-}4S]^{2+}$ cluster-containing FNR under anaerobic conditions and become exposed upon loss of the $[4Fe\text{-}4S]^{2+}$ cluster and dissociation of the FNR dimer. A simple model for explaining this is that perhaps one or both of the ClpXP recognition sites are buried in the interaction surface and thus are exposed upon dissociation of the FNR dimer. However, several existing observations do not support this assumption. First, apo-FNR was degraded at an unusually slow rate under aerobic conditions (half-life of ∼115 ± 8 min determined by the pulse-chase experiment).[9] Second, several analyses, including the limited protease digestion, revealed that there were no significant conformational changes between the $[4Fe\text{-}4S]^{2+}$ cluster-containing FNR dimer and the clusterless FNR monomer, with only slightly greater sensitivity of the C-terminal of apo-FNR to the limited protease digestion than that of the $[4Fe\text{-}4S]^{2+}$ cluster-containing FNR.[14,21] Third, alanine substitution of amino acids 239−241 and 242−244 in apo-FNR caused further accelerated degradation and a significant decrease in FNR protein levels in *E. coli* under aerobic conditions. All these observations suggest that the ClpXP recognition signals of FNR are not readily exposed even in the apoprotein and the accessibility by the ClpXP must be mediated by a certain element. We speculate this element is the region constituted by amino acids 239−244, because alanine substitution in this region, but not those located in other regions of FNR characterized so far, causes a decreased FNR protein level and an accelerated degradation compared to those of WT under both anaerobic and aerobic conditions. Our

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CD analysis demonstrating that replacing amino acids 239−241 with three alanines caused alterations in the secondary structures of the WT and an O_2 stable dimer FNR D154A also supports this speculation.

However, the readiness and strength of this determinant to protect the proximal ClpXP recognition site of residues 249 and 250 must be dependent on $[4Fe\text{-}4S]^{2+}$ cluster binding and its induced FNR dimerization, as only the clusterless apo-FNR is subject to degradation in vivo, although with a relatively slow rate. A more feasible model for explaining these observations is that perhaps it is the region of residues 239−244 rather than the ClpXP recognition sites themselves that is buried and strengthened at the extended interface between the FNR subunits and undergoes conformational changes that lead to the exposure of the VA249−250 ClpXP site upon dissociation of the FNR dimer (Figure 8). This speculation is supported by the conservation of the $L^{239}XXL^{242}XG^{244}$ stretch among the FNR orthologs containing the Cys-X_2-Cys-X_5-Cys signature for cluster binding, and the correlation of the conservation with that of I151 and D154, two important amino acids that mediate $[4Fe\text{-}4S]^{2+}$ cluster-induced dimerization. Consistent with this speculation, introducing the D154A substitution, which promotes FNR dimerization even in the absence of the $[4Fe\text{-}4S]^{2+}$ cluster, partially rescues the decreased activity and protein level of LAQ239−241A_3 (data not shown). Furthermore, the presence of an extended subunit−subunit contact in addition to that solely between the dimerization helix was also observed in other proteins such as apo-CRP, which is a dimer that is more stable than that of the effector-bound CRP. Three-dimensional crystal structures revealed that in apo-CRP a substantive contact between the two D-helices located at the C-terminal DNA binding domain of the two CRP subunits was also observed in addition to the interaction between the two C-helices,[28,29] which is present in both apo-CRP and effector-bound CRP. This evidence provides a likelihood for the amino acids 239−241 to form an extended contact in the FNR dimer that contributes to the protection of the ClpXP recognition sites from exposure.

As for the N-terminal recognition signal, although previous studies suggested that amino acids 5−11 specify this recognition site, our studies showed that deletion of these amino acids did not cause an elevated intracellular level of FNR in the cell. Rather, it caused an ∼50% decrease in both the transcription activity and the FNR protein level. Moreover, alanine block substitution of residues 5−7 and 8−10 had different effects on the activity and FNR protein level in the cell (Figure 2A). While the KRI5−7A_3 substitution caused a reduced activity and a reduced protein level, the IRR8−10A_3 substitution caused an increase in the activity and protein level. Because studies have shown that the N-terminal recognition site often favors positively charged residues when two sites are required for ClpXP recognition,[11] we suggest that perhaps amino acids 9 and 10 (RR) are largely responsible for the recognition and binding by the ClpXP protease in this site. Our results demonstrating that replacing RR9−10 with DD resulted in a protein level higher than that of WT (Figure 5) further support this notion. The observed reduction in the activity and protein level of the mutant KRI5−7A_3 perhaps is due to its effect on the proximal N-terminal recognition site RR9−10, analogous to the roles of residues 239−244 to the C-terminal site VA249−250. However, because substitution of amino acids 5−7 with alanine did not cause a decrease in the level of FNR protein as significant as that seen in the alanine substitution of

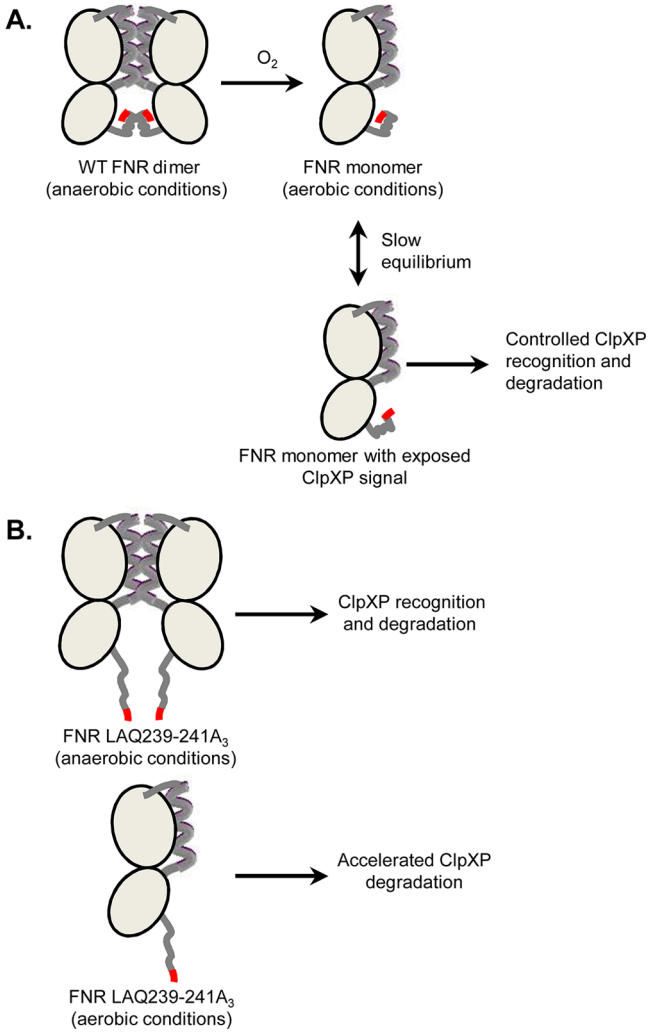

Figure 8. A model demonstrating the role of amino acids 239−244 in mediating the ClpXP-dependent proteolysis of FNR. (A) An extended structural contact formed among amino acids 239−241 of the two FNR subunits is buried and stabilized in the dimerization interface of FNR dimer under anaerobic conditions, which prevents the exposure of the C-terminal ClpXP recognition site (red). With the switch to the aerobic conditions, the stability of this extended structural contact is decreased by dissociation of the FNR dimer, resulting in an equilibrium between the ClpXP-exposed and unexposed form. The FNR monomer with the exposed ClpXP signal is recognized and degraded by the ClpXP protease. (B) Alanine substitution of LAQ239−241 causes conformational changes, leading to certain exposure of the C-terminal ClpXP signal even in the FNR dimer under anaerobic conditions. Under aerobic conditions, the alanine substitution mutation causes a full exposure of the ClpXP signal, resulting in its rapid degradation under this condition.

239−244, especially residues 239−241, and no similarity or conservation of these amino acids is observed in FNR orthologs, we propose that the readiness of FNR degradation is largely mediated by amino acids 239−244 through their effect on the C-terminal ClpXP recognition site.

It is not uncommon that proteolysis of the key regulatory factors is exquisitely controlled in both prokaryotic and eukaryotic cells, such as heat shock regulator σ^H and general stress regulator σ^S.[26,30] As seen for FNR, both their levels and activities are modulated by multiple factors at multiple levels to exert a fine-tuned transcription regulation of their target genes

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in response to environmental and cellular stresses. The exquisitely regulated proteolysis of FNR perhaps is important to the physiology of *E. coli* and other facultative bacteria in their natural niches in which O_2 levels may fluctuate constantly. *E. coli* requires a proper level of intracellular FNR protein reservoir, so that in the case of an instant O_2 deprivation, cells can rapidly utilize the already present apoproteins to convert it to an active form before the initiation of de novo FNR protein synthesis. To this end, FNR must have a very fine-tuned degradation rate even under aerobic conditions to maintain a proper level of FNR protein, which should be sufficiently high that there are sufficient FNR proteins to be activated when cells are instantly deprived of O_2 and sufficiently low that no nonspecific dimerization occurs under aerobic conditions by the high level of FNR protein. This highlights the important role of amino acids 239−244 we identified in this study that links the status of protein dimer−monomer conversion to the specific and regulated ClpXP degradation of the protein, thus constituting an added modulation on the O_2 sensing capacity of FNR family proteins.

Although extensive mutagenesis studies of FNR[14,16,22,31−34] have been performed and valuable information about the function and regulation of this global transcription factor have been obtained, much remains unknown, such as the detailed pathway and kinetics of the dimer−monomer conversion and their correlation with the mediated proteolysis of FNR. Answering these questions perhaps will rely on the X-ray crystal structures of both the Fe−S cluster-containing FNR and the FNR apoprotein.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Three additional supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This paper is dedicated to the memory of Dr. Christian R. H. Raetz, a beloved mentor who passed away on August 16, 2011.

■ ABBREVIATIONS

FNR, fumarate nitrate reduction; CRP, cAMP receptor protein; WT, wild type; CD, circular dichroism.

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