Dissecting the Role of the N-Terminal Region of the Escherichia coli Global Transcription Factor FNR
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The role of the N-terminal region of the transcription factor FNR, which immediately precedes the first ligand (Cys20) of the [4Fe-4S] cluster, was investigated. We found that truncation mutants that removed residues 2 to 16 and 2 to 17 had wild-type levels of FNR protein but surprisingly altered O2 regulation.

FNR is a global transcription regulator that mediates the transition from an aerobic to an anaerobic lifestyle in many facultative anaerobes (9, 10). In Escherichia coli K-12, FNR senses and responds to O2 deprivation by altering the transcription of hundreds of genes (3, 5, 6, 18). The ability of FNR to function as an O2 sensor is dependent on its [4Fe-4S] cluster (7, 8, 14). Under anaerobic conditions, FNR exists as a homodimer, with each monomer containing a [4Fe-4S]2+ cluster, which promotes dimerization and site-specific DNA binding and, accordingly, gene regulation (14). Upon a switch to aerobic conditions, O2 converts the [4Fe-4S]2+ cluster to a [2Fe-2S]+ cluster, resulting in the disassembly of the FNR dimer and the subsequent loss of site-specific DNA binding (8, 14). Four essential cysteine residues, Cys20, Cys23, Cys29, and Cys122, are required to ligate the [4Fe-4S]2+ cluster to FNR, and they are located in the N-terminal half of the protein (1, 15, 19). However, little is known about the function of the 19 amino acid residues that precede the cysteine ligands.

FNR belongs to the CRP/FNR superfamily of transcription factors that have a conserved helix-turn-helix motif in the C-terminal domain required for DNA binding and an N-terminal effector binding domain that in cyclic AMP (cAMP) receptor protein (CRP) binds cAMP. However, only a subset of this superfamily appears to bind cAMP; rather, this N-terminal fold seems to be involved in signaling of many small molecules, such as O2, CO, aromatic compounds, and oxoglutarate, etc. (12). In addition, O2 signaling in FNR requires an additional 29 amino acids at its N terminus because three (Cys20, 23, and 29) of the four Fe-S cluster ligands are located within this element. While mutagenesis and biochemical experiments have clearly established the role of Cys20, Cys23, and Cys29 in ligation of the cluster, less is known about the role of the first 19 residues, even though they are conserved in FNR orthologs present in closely related species (Fig. 1A). Residues 5 to 11 were previously identified as a ClpXP protease binding site, which functions in the regulated turnover of the apoferritins of FNR under aerobic conditions (16). Protease analysis demonstrated that the N-terminal region is susceptible to limited trypsin digestion, suggesting that this region is either unstructured or surface exposed (17).

To dissect the role of the first 19 amino acid residues of FNR, we constructed a series of N-terminal truncation mutants (Fig. 1B) by site-directed mutagenesis of pPK823 (13) and studied the properties of these mutant proteins by measuring the β-galactosidase activity produced from strains containing lacZ transcriptional fusions to FNR-dependent promoters. The amount of β-galactosidase activity produced from anaerobically grown strains containing either dmsA-lacZ (data not shown) or narG-lacZ (Fig. 2) showed that removal of residues 2 and 3 from FNR (FNRΔ2-3) had no effect on transcription activation of either the narG or the dmsA promoter, whereas removal of residues 2 to 8 (FNRΔ2-8) reduced FNR activity ~8-fold. However, elimination of just seven more residues (FNRΔ2-15) restored FNR activity to levels 1.5-fold greater than those for full-length FNR. This unexpected observation led us to further characterize the role of residues 8 through 19.

Truncation of residues 2 to 10, 2 to 12, or 2 to 13 of FNR caused a complete loss of activation of the narG promoter (Fig. 2). However, elimination of just one additional amino acid, Gly14, partially restored transcription activity such that the activity was now 50% relative to that of the full-length protein. Unexpectedly, FNR protein levels in strains containing truncation of residues 2 to 10, 2 to 12, or 2 to 13 were found to be greatly reduced (>20-fold) by Western blot analysis (Fig. 2), and FNR transcription activity was largely correlated with the amount of FNR protein (Fig. 2). It seems unlikely that these truncation mutants have accelerated the ClpXP-dependent proteolysis of FNR, since neither FNRΔ2-12 nor FNRΔ2-13 contains the N-terminal ClpXP binding site (residues 5 to 11), and the second ClpXP binding site (residues 249 and 250) is not sufficient to target FNR for ClpXP-dependent proteolysis (16). It also seems unlikely that decreased transcription can explain the reduction in protein levels, since transcription of these mutant genes is driven by plasmid sequences and should be unaffected by the truncation mutations. Thus, we suggest that perhaps another proteolytic site becomes unmasked in these mutants, resulting in their proteolysis.

In contrast, truncation of amino acids 2 to 16 and 2 to 17 (Fig. 2) showed slightly increased levels of β-galactosidase activity expressed from the narG promoter-lacZ fusion, similar to that found with the truncation of residues 2 to 15. In addition, the FNR protein levels in these truncation mutants were the...
same as those in full-length FNR, indicating that the element that leads to the posttranscriptional reduction in FNR protein levels was removed.

The additional removal of Ile18 (FNRΔ2-18) decreased FNR activity ~3-fold (Fig. 2), with a corresponding decrease in FNR protein levels, similar to what was found for FNRΔ2-14, whereas the additional elimination of His19 (Fig. 2), adjacent to the first cluster ligand, Cys20, abolished FNR activity, although protein levels were reduced only ~3-fold. The complete loss in FNR activity of FNRΔ2-19 is similar to what has been observed for FNR mutants containing substitutions for the Cys ligands. This led us to consider that Cys20 was removed by methionine aminopeptidase because of its preference for cleaving methionine as well as the following amino acid residue when it is Ala, Cys, or Ser (4). In support of the idea that His19 has a less critical function in FNR than Cys20, we found that replacement of His19 in FNRΔ2-18 with Val does not change the activity of this mutant significantly (Fig. 2). However, the basic side chain of His19 is likely to play a small role since replacement with Tyr but not the similarly charged amino acid Arg decreases FNR activity about twofold (15). Therefore, there seems to be only a slight preference for a basic residue immediately preceding Cys20.

Since previous studies of FNR identified amino acid substitutions in the N-terminal region that allowed FNR to be active in the presence of O2 (FNR* mutants) (1, 11, 15), we investigated whether any of the truncations altered the response of FNR to O2 by assaying FNR activity under aerobic growth conditions. As expected, the previously characterized FNR* mutants FNR(D154A) and FNR(L28H) showed increased expression of the narG (Fig. 3A) promoter and another FNR-dependent promoter, PpydfZ (6) (Fig. 3B), under aerobic growth conditions. The truncation mutant of FNR residues 2 to 15 also increased the activity of FNR under aerobic conditions.

FIG. 1. (A) Sequence alignment of the first 29 N-terminal amino acids of FNR proteins from selected gammaproteobacteria. Rows: 1, *Escherichia coli* K-12; 2, *Salmonella enterica* serovar Typhimurium; 3, *Pectobacterium atrosepticum*; 4, *Yersinia pestis*; 5, *Photobacterium luminiscens* subsp. laumondii; 6, *Vibrio fischeri*; 7, *Shewanella oneidensis*; 8, *Actinobacillus pleuropneumoniae*; 9, *Pseudomonas aeruginosa*; and 10, *Bordetella pertussis*. Bold letters indicate the Cys residues that have been shown to be the ligands to the [4Fe-4S] cluster in *E. coli* FNR.

(B) Diagram of the FNR truncation mutants constructed from pPK823 (13) by site-directed mutagenesis; the DNA sequence of the mutant variants was confirmed at the University of Wisconsin Biotechnology Center. Each bar represents the specific residues of FNR that were deleted.

![FIG. 2](http://jb.asm.org/) Effect of N-terminal truncation mutants on FNR activity and protein levels. β-Galactosidase activity (reported in Miller units) was determined as previously described (13) from derivatives of strain RZ8480 (narG-lacZ), transformed with vector plasmid (pET11a) or plasmid derivatives encoding N-terminal truncation mutants, which were grown under anaerobic conditions in M9 minimal medium with 0.2% (wt/vol) glucose, 10 μM ferric ammonium citrate, 1.4 mM KNO₃, and 0.2 μM ammonium molybdate. The values shown for β-galactosidase activity (gray bars) are means of results from three independent experiments, expressed as percentages relative to the activity for full-length FNR (the average activity of wild-type FNR [expressed from pET11a] was 250 Miller units, and the average error from triplicate samples was less than 10%). Cells were also subjected to Western blot analysis (16) to measure the amount of full-length FNR and various FNR truncation mutants after growth under the same conditions as those for the β-galactosidase assays. Proteins were detected with UV, and the amounts of FNR were quantified using Molecular Dynamics ImageQuant software and expressed as percentages relative to the level for full-length FNR (black bars). Values shown are means of results from duplicate experiments.
lyzed by mass spectrometry. Only one major cleavage product was treated with trypsin under anaerobic conditions and anaerobic conditions, and analysis of the visible absorbance spectrum confirmed that it had the same type of Fe-S cluster as the full-length protein. The increased stability of the cluster is consistent with that had the same type of Fe-S cluster as the full-length protein. The increased stability of the cluster is consistent with the overall structure of FNRΔ2-15 is not significantly altered. In addition, this mutant protein may prove useful for crystallography experiments since removal of this protease-accessible region may improve crystallization of FNR.

The MS data also showed that isolated FNRΔ2-15 contains only residues 17 to 250 of FNR, indicating that the N-terminal Met and Cys16 residues have been removed (Table 1), most likely as a result of cleavage from methionine aminopeptidase because of its preference for cleaving Cys following the N-terminal Met residue (4). A parallel set of truncation mutants that initiated with Met-Val was constructed to evaluate any possible effects of the first set of mutants resulting from cleavage of the second amino acid. Overall, the insertion of Val did not alter the activity or protein levels of the truncation mutants except for FNRΔ2-19 (described above) and FNRΔ2-15 except for FNRΔ2-19 (described above) and FNRΔ2-15. Insetion of a Val residue preceding Cys16 [FNRΔ2-19] resulted in a mutant protein in which both the protein level and β-galactosidase activity produced from the narG-lacZ fusion under anaerobic conditions are similar to those for full-length protein (Fig. 2), suggesting that removal of Cys16 in FNRΔ2-15 (shown by mass spectrometry; see above), FNRΔ2-16, and FNRΔ2-17 may partially explain their increased activity. In support of this notion, replacement of Cys16 with Val in FNRΔ2-15 has activity similar to that of FNRΔ2-19, which was observed with FNRΔ2-15, which corresponds to the cleavage product at Arg247 observed with the full-length protein (Table 1). The other two residues that lead to trypsin digestion products in the full-length protein, Arg9 and Arg10, are not present in the truncation mutant, and accordingly, these sites of cleavage were not detected. Thus, we conclude that the overall structure of FNRΔ2-15 is not significantly altered. In addition, this mutant protein may prove useful for crystallography experiments since removal of this protease-accessible region may improve crystallization of FNR.

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It is also interesting to note that the N-terminal region is conserved in closely related FNR orthologs (Fig. 1A, rows 1 to 6), suggesting a similar function. However, this degree of conservation is not observed in orthologs from more-distantly related species (Fig. 1A, rows 7 to 10). In particular, the Pseudo-
**TABLE 1. Assignments of mass spectroscopy analysis on limited trypsin digestion of full-length FNR or FNRΔ2–15**

| FNR sample                  | Peak no. | Molecular mass | Trypsin cleavage site(s) | Corresponding polypeptides |
|-----------------------------|----------|----------------|--------------------------|----------------------------|
| Full-length                 | 1        | 27966          | 27967                    | 1–25                       |
| Limited trypsin digested    | 1        | 26828          | 26829                    | Arg9 10–250                |
|                             | 2        | 26672          | 26673                    | Arg10 11–250               |
|                             | 3        | 26544          | 26545                    | Arg9, Arg247 10–247        |
|                             | 4        | 26388          | 26389                    | Arg10, Arg247 11–247       |
| Δ2–15                       | 1        | 26230* (26126*) | 26126                    | 17–250                     |
| Limited trypsin digested Δ2–15 | 1     | 25842          | 25842                    | Arg247 17–247 17–247       |

* Limited trypsin digestion and mass spectroscopy were performed as described previously (17). Mass spectra were obtained at the Biotechnology Center of the University of Wisconsin—Madison.

b Predicted non-methionine aminopeptidase cleavage product.

c Calculated methionine aminopeptidase cleavage product.

*monas aeruginosa* FNR ortholog, ANR, has a much shorter and divergent N terminus, raising the question of whether this region has a similar function in all orthologs.

In summary, removal of N-terminal amino acid residues 2 to 16 and 2 to 17 increases FNR activity under aerobic conditions, suggesting that this N-terminal region actively contributes to the lability of the [4Fe–4S] cluster of FNR to O₂. Previous characterization of FNR* mutants within this region suggested that some of the amino acid substitutions may act by stabilizing the cluster, perhaps by forming hydrogen bonds (2). However, the results in this study suggest that some of these mutants may act by removing interactions that act to increase the lability of the cluster to O₂. Future work will focus on these possibilities.

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