Role of Activating Region 1 of *Escherichia coli* FNR Protein in Transcription Activation at Class II Promoters*

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FNR is an *Escherichia coli* transcription factor that activates gene expression in response to anaerobiosis at a large number of promoters by making direct contacts with RNA polymerase. At class II FNR-dependent promoters, where the DNA site for FNR overlaps the −35 element, activating region 1 of FNR is proposed to interact with the C-terminal domain of the RNA polymerase α-subunit. Using a model class II FNR-dependent promoter, FF(−41.5), we have performed *in vivo* and *in vitro* experiments to investigate the role of this interaction. Our results show that FNR, carrying substitutions in activating region 1, is compromised in its ability to promote open complex formation and thus to activate transcription. Abortive initiation assays were used to assess the contribution of activating region 1 of FNR to open complex formation. A new method for the purification of the FNR protein is also described.

Many bacterial gene regulatory proteins activate transcription by making direct contact with RNA polymerase holoenzyme (RNAP). A large number of these activators contact RNAP via the C-terminal domain of the α-subunit (αCTD) (1). One of the best studied examples of this type of activator is the *Escherichia coli* cAMP receptor protein, CRP (reviewed in Refs. 2 and 3). In response to glucose starvation, CRP is triggered by cAMP and activates transcription initiation at more than 100 different promoters. In its active form, CRP binds as a dimer to specific DNA sequences found at target promoters; the consensus site is a 22-base pair sequence that is organized as an 11-base pair inverted repeat. At CRP-dependent promoters, CRP activates transcription by making several direct contacts with RNAP. One of these contacts involves the interaction of a single surface-exposed β-turn of CRP (residues 156–164) with αCTD. This region in CRP is known as activating region 1 (AR1). The role of AR1 of CRP is to enhance open complex formation at target promoters simply by increasing the initial binding of RNAP (reviewed in Ref. 2).

The FNR protein is another global activator of gene expression in *E. coli*, which regulates transcription initiation in response to oxygen starvation (4, 5). FNR belongs to the same family of transcription factors as CRP, and the two proteins have related amino acid sequences (6). Although the high resolution structure of the FNR protein has not been determined, several crystallographic structures for CRP have been solved (7–9) and sequence alignments suggest that FNR and CRP have similar three-dimensional structures (4, 6). Like CRP, FNR regulates transcription as a dimer and recognizes a 22-base pair binding sequence at target promoters. However, dimerization of FNR is triggered by the anaerobic acquisition of a [4Fe-4S]^{2+} center in each FNR subunit (10–12). By analogy to CRP, during transcription initiation at FNR-dependent promoters it is proposed that FNR interacts with the αCTD of RNAP. Genetic analysis suggests that the amino acid side chains involved in this interaction are located in three adjacent surface-exposed loops, residues 71–75, 116–121, and 184–192 (13). These loops form an extended surface of FNR, also known as AR1, which is much larger than AR1 of CRP (although AR1 of FNR and CRP do overlap, the 184–192 loop of FNR is equivalent to the β-turn containing residues 156–164 in CRP). The key residues in AR1 of FNR, which are likely to provide the crucial side chains for the FNR-αCTD interaction, were identified as Thr118 and Ser187 (alanine substitutions at these positions reduce transcription activation by FNR) (13).

Most naturally occurring FNR-dependent promoters contain a DNA site for FNR that overlaps the −35 element (promoters organized in this way are known as class II promoters) (5). Previous studies with class II FNR-dependent promoters showed that AR1 of FNR is functional on the upstream subunit of the FNR dimer, and thus it is this subunit that is thought to contact αCTD (14). The aim of this work was to investigate the role of AR1 of FNR during transcription activation using *in vitro* approaches with the model class II FNR-dependent promoter, FF(−41.5) (described in Ref. 14). To study the FNR protein under aerobic conditions in *vitro*, the D154A variant of FNR (denoted FNR*) (15) was used. The D154A substitution stabilizes FNR in its active dimeric form, thereby relieving the need for the [4Fe-4S]^{2+} center (16). Our results show that, as for CRP, substitutions in AR1 of FNR cause clear defects in open complex formation. Kinetic analysis suggests that AR1 of FNR is involved in promoting isomerization from the transcriptionally inactive closed complex to the transcriptionally active open complex.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—pFNR T118A/S187A was constructed by site-directed mutagenesis using PCR with pFNR T118A as template DNA (13). pFNR derivatives encoding FNR*, FNR T118A, FNR* S187A, and FNR* T118A/S187A were also constructed by PCR using pFNR, pFNR T118A, pFNR S187A, and pFNR T118A/S187A, respectively, as templates. PCR products from these reactions were cut with *Bam*HI and *Hind*III, and the resulting fragments were cloned into...
purified BamHI-HindIII vector from pFNR. The NcoI and BglII restriction sites, which were used to clone the different fur derivatives into the pQ6E0 C-terminal His tag overexpression vector (supplied by QIAGEN), were generated by PCR. The resulting DNA was cut with NcoI and BglII, and fragments were cloned into the purified NcoI-BglII vector from pQ6E0 so that the fur gene and the C-terminal His tag were in frame.

In Vivo Assays—pFNR derivatives were transformed into competent JRG1728 Δ lac Δ fnr cells (17) containing the FF(−41.5):lacZ fusion carried by the plasmid, pMRW50 (described in Ref. 18). Transformants were grown either aerobically or anaerobically at 37 °C in L-broth supplemented with final concentrations of 0.4% (v/v) dextrose, 50 μg/ml ampicillin, and 35 μg/ml tetracycline. β-Galactosidase levels were determined using the Miller protocol (19) to quantify transcription from the FF(−41.5) promoter.

Overexpression and Purification of FNR* Derivatives—As a host background for the overexpression of the FNR* derivatives, the strain M15 (derived from E. coli K12; supplied by QIAGEN) carrying the plasmid pREP4 (derived from pACV and encoding constitutively expressed LacI; supplied by QIAGEN) was used. Cells were transformed with pQ6E0 derivatives encoding different C-terminally His-tagged FNR* proteins, and transformants were grown at 37 °C in 100 ml of L-broth with appropriate antibiotics until cultures reached an A600 of 0.5–0.6. Overexpression of the His-tagged proteins was then induced by the addition of 0.1% (v/v) isopropyl-1-thio-β-D-galactopyranoside for 1 h. Cells were harvested, and pellets were resuspended in 1 ml of buffer containing 8% btx, 0.2 M NaCl, 2% Triton X-100, 50 mM glycerol, 100 mM EDTA, 0.1 mM dithiothreitol, and 50 μg/ml bovine serum albumin. After preincubation at 37 °C for 10 min, reactions were started by adding 10 μl of RNAP in binding buffer (this gave final reaction conditions of 2 mM template DNA, 350 mM FNR* derivative, 50–150 mM RNAP, 0.5 mM ApU, 50 μM UTP, and 0.75 μCi [α-32P]UTP). At regular time intervals, 2-μl samples were removed from these reactions and mixed with 20 μl of stop buffer (20 mM EDTA, 80% (v/v) deionized formamide, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophen blue). The reaction product, ApUpU, and unincorporated UTP were separated on 20% denaturing polyacrylamide gels and quantified using a Molecular Dynamics PhosphorImager and the software ImageQuant, v3.3 (as described in Ref. 24). The kinetic constants for transcription initiation at FF(−41.5) were calculated by measuring the rate of ApUpU production over a range of RNAP concentrations and by plotting the lag time (τ) of these reactions against the reciprocal of RNAP concentration.

Heparin Challenge Experiments—Template DNA was prepared by PCR amplification of the FF(−41.5) promoter. Binding reactions contained DNA, purified FNR* or mutant derivatives, and RNAP in 20 μl of binding buffer (5% (v/v) glycerol, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM diethiothreitol, 50 μg/ml bovine serum albumin). Reactions were preincubated at 37 °C to allow open complexes to form. After 30 min, open complexes were challenged with 10 μl of heparin (three concentrations were used to give final concentrations of 10, 80, or 150 μg/ml). At appropriate time intervals up to 70 min, 3-μl samples were removed from the challenged complexes and 3 μl of ApU (and [α-32P]UTP were added (this gave final reaction conditions of 2 mM template DNA, 350 mM FNR* derivative, 100 mM RNAP, 0.5 mM ApU, 50 μM UTP, and 0.75 μCi [α-32P]UTP). After incubation at 37 °C, 2 μl of each reaction was removed and added to 3 μl of stop buffer. The reaction product, ApUpU, and unincorporated UTP were separated on 20% denaturing polyacrylamide gels and quantified using a Molecular Dynamics PhosphorImager and the software ImageQuant, v3.3 (as described in Ref. 24). The fraction of the open complex remaining was calculated from the amount of ApUpU produced. The decay rate of this fraction was calculated for each heparin concentration, and the backward rate constants for isomerization (k−) of FF(−41.5) in the presence of FNR* or FNR* T118A/S187A were determined by extrapolating these data to a zero heparin concentration.

RESULTS

In Vivo Transcription Assays—The starting point of this work was the pFNR plasmid that encodes FNR. A derivative of pFNR, pFNR*, encoding FNR with the D154A substitution, known to confer FNR activity in the presence of oxygen (15), was constructed. The aim of this work was to investigate the role of AR1 in transcription activation by FNR at class II promoters. Thus, three derivatives of pFNR* were constructed that encoded alanine substitutions at either or both of the key residues in AR1: pFNR* T118A, pFNR* S187A, and pFNR* T118A/S187A. To check that FNR* was able to function aerobically and to investigate the effects of the different AR1 substitutions when combined with D154A, transcription activation was measured in vivo at the FF(−41.5) promoter. The FF(−41.5) promoter is an artificial class II FNR-dependent promoter that contains a consensus DNA site for FNR centered between 41 and 42 base pairs upstream of the mleR transcription start site (i.e. at position −41.5). The Δ lac Δ fnr strain, JRG1728, carrying the FF(−41.5):lacZ fusion in pMRW50 (14), was transformed with the four different pFNR* derivatives. Levels of transcription from FF(−41.5) were determined by measuring β-galactosidase expression in cells that had been grown aerobically or anaerobically. The data in Table I show that in the absence of FNR*, under both aerobic and anaerobic conditions, transcription from the FF(−41.5) promoter is very low. Expression from FF(−41.5) is clearly activated by FNR* under both aerobic and anaerobic conditions, but FNR* variants carrying either or both of the alanine substitutions in AR1 are compromised in their ability to activate transcription. Thus, FNR* is able to activate transcription under aerobic conditions and activation is disrupted by the different AR1 substitutions. These data suggest that these FNR derivatives could be used in in vitro studies at the FF(−41.5) promoter.
TABLE I

In vivo activity of FFR(−41.5) with FNR* and different derivatives carrying alanine substitutions in AR1

The β-galactosidase activities (Miller units) were measured in JRG1728 transformants carrying FFR(−41.5) in the lac expression vector, pRW50, and expressing different FNR* derivatives. Cultures were grown either aerobically or anaerobically. Average values are shown for three independent assays; in each case the S.D. fell within 20% of the mean.

| FNR derivative | β-Galactosidase activities |
|----------------|---------------------------|
|                | Aerobic                  | Anaerobic                |
| FNR*           | 740                       | 4340                     |
| FNR* S187A     | 360                       | 3190                     |
| FNR* T118A     | 330                       | 2300                     |
| FNR* T118A/S187A| 250                       | 1660                     |
| No FNR         | 50                        | 170                      |

**Purification and Properties of FNR* and FNR* Carrying AR1 Substitutions**—To investigate the effects of substitutions in AR1 of FNR in vitro, His-tagged FNR*, FNR* T118A, FNR* S187A, and FNR* T118A/S187A were purified from cell lysates using nickel affinity chromatography. Typically, culture volumes of 100 ml yielded 300–400 mg of soluble protein, of which 20–25% was His-tagged FNR* protein. Affinity chromatography yielded 2–8 mg of the different His-tagged FNR* derivatives, and the protein preparations were estimated to be 90% pure by SDS-polyacrylamide gel electrophoresis. All of the buffers used throughout this procedure contained 0.75 M NaNO₃, which was found to improve the solubility and stability of the FNR* proteins. After anaerobic reconstitution by the method of Green et al. (20), His-tagged FNR* and His-tagged FNR* T118A/S187A had optical spectra indistinguishable from those of unaltered FNR. Assuming that all the absorbance at 420 nm was because of the [4Fe-4S] form of FNR, the FNR molar extinction co-efficient (ε₄₂₀ = 13,300 M⁻¹ cm⁻¹) was used to calculate that our FNR preparations contained 0.95 clusters/FNR subunit. Exposure of the reconstituted proteins to air yielded 2–8 mg of the different His-tagged FNR* derivatives, and the protein preparations were estimated to be 90% pure by SDS-polyacrylamide gel electrophoresis. All of the buffers used throughout this procedure contained 0.75 M NaNO₃, which was found to improve the solubility and stability of the FNR* proteins. After anaerobic reconstitution by the method of Green et al. (20), His-tagged FNR* and His-tagged FNR* T118A/S187A had optical spectra indistinguishable from those of unaltered FNR. Assuming that all the absorbance at 420 nm was because of the [4Fe-4S] form of FNR, the FNR molar extinction co-efficient (ε₄₂₀ = 13,300 M⁻¹ cm⁻¹) was used to calculate that our FNR preparations contained 0.95 clusters/FNR subunit.

**In Vitro Transcription Assays**—In vitro transcription assays were used to investigate the effects of different substitutions in AR1 of FNR. Fig. 1 shows a typical result using the FFR(−41.5) promoter cloned upstream of the λ oop terminator in a supercoiled plasmid, in the presence of purified RNAP and the different purified FNR* variants. Transcription initiation from FFR(−41.5) is clearly activated by FNR*. This activation is reduced by the different alanine substitutions in AR1, confirming the effects observed in vivo, and showing that substitutions in AR1 compromise the ability of FNR* to activate transcription at the FFR(−41.5) promoter.

**DNase I and Potassium Permanganate Footprinting Experiments**—To study open complex formation in the presence of FNR* or FNR* carrying substitutions in AR1, we used DNase I and potassium permanganate footprinting. Saturating amounts of FNR* or FNR* T118A/S187A (350 nM in both cases) and a range of RNAP concentrations were used to form open complexes at the FFR(−41.5) promoter. The DNase I footprints (Fig. 2) show that, when either FNR* or FNR* T118A/S187A is incubated with the promoter fragment alone, a region centered at position −41.5, relative to the transcription start point, is protected (lanes c and k). Thus both FNR* derivatives recognize and bind to the FNR target site. With FNR*, increasing concentrations of RNAP result in increasing protection of the promoter region both upstream and downstream of the FNR binding site (lanes d–i). Based on footprinting studies of CRP...
and RNAP at class II CRP-dependent promoters, the upstream protection can be attributed to the CTD of RNAP (25, 26). Footprints with FNR* T118A/S187A also show increasing protection with increasing RNAP concentrations (lanes i–q), although significantly less protection is seen in comparison to the footprints with FNR*. Interestingly, the pattern of protection of ternary complexes containing either FNR* or FNR* T118A/S187A is the same. This is in contrast to the situation observed at class II CRP-dependent promoters, where substitutions in AR1 of CRP induce changes in the DNase I footprint in the region of the CRP–aCTD interface (26). Strikingly, in the experiment described here, no protection is seen with RNAP alone, even at 500 nM (lane j), showing that RNAP alone cannot form stable complexes at the FF(−41.5) promoter.

Potassium permanganate footprints show the appearance of bands because of unpaired thymine bases that result when the DNA duplex is unwound near the transcript start point during open complex formation. The results in Fig. 3A show that, at FF(−41.5), bands because of oxidation of these thymine bases appear at positions +1, −2, −8, −9, and −11, with both FNR* (lanes c–h) and FNR* T118A/S187A (lanes j–o), as RNAP is added. However, as the RNAP concentration is increased, the intensity of the bands in lanes containing FNR* T118A/S187A is significantly lower than in the lanes containing FNR* (see Fig. 3B). Note that thymine-sensitive bands are not seen with RNAP alone (lane i), implying that promoter opening is completely FNR-dependent.

Abortive Initiation Assays—The in vitro footprint experi-

| FNR derivative | $K_b$ (M$^{-1}$) x 10$^4$ | $k_5$ (s$^{-1}$) x 10$^5$ | $k_b k_5$ (M$^4$ s$^{-5}$) x 10$^6$ |
|----------------|-----------------|-----------------|-----------------|
| FNR*           | 3.75            | 1.53            | 5.73            |
| FNR* T118A S187A | 6.86            | 0.52            | 3.55            |
FNR*-dependent open complexes at FF(-41.5). The observed rate constant for dissociation of RNA polymerase from the FF(-41.5) promoter is plotted as a function of heparin concentration. Values for the backward rate constant for isomerization ($k_{2}$) in the absence of heparin, were extrapolated to be $8.4 \times 10^{-6}$ s$^{-1}$ for FNR$^{a}$ and $6.3 \times 10^{-3}$ s$^{-1}$ for FNR$^{a}$ T118A/S187A. The observed rate constant for dissociation of RNA polymerase from the FF(-41.5) promoter was measured in the presence of FNR$^{a}$ T118A/S187A at FF(-41.5). The rate of ApUpU production at FF(-41.5) was clearly shown to be more than 80-fold lower than with FNR$^{a}$. This is in contrast to the situation with CRP, where AR1 is responsible for contacting an equivalent of RNA polymerase to promoter DNA (2). Thus, whereas both CRP and FNR function by interacting directly with αCTD, the consequences of these interactions appear to differ. This hypothesis is supported by the observation that FNR fails to recruit purified αCTD in electromobility shift assays (data not shown), unlike CRP, which binds α cooperatively (28). Interestingly, the surface of FNR that is responsible for contacting αCTD is much larger than the equivalent surface of CRP (2, 13). Further studies are needed to determine the individual contributions of the subregions of AR1 to the process of transcription activation.

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DISCUSSION
Many bacterial transcription factors activate transcription by making direct contact with the αCTD of RNAP. For example, at CRP-dependent promoters the AR1–αCTD interaction helps to recruit RNAP to the promoter DNA (2). Previous studies have suggested that FNR also functions by interacting with αCTD and have identified Thr$^{118}$ and Ser$^{187}$ as the crucial side chains of the AR1 equivalent in FNR (13, 14). In this work, we have used in vitro studies to show that FNR carrying the T118A and S187A substitutions is indeed defective in transcription activation and is less able to promote the formation of open complexes. Using abortive initiation assays, we have assessed the role of AR1 of FNR. Although the large errors prevent an accurate estimation of $k_{2}$ and $K_{p}$, the results suggest that AR1 of FNR plays a role in the transition from a closed to an open complex (at least at the FF(-41.5) promoter). This is in contrast to the situation with CRP, where AR1 has been reported to function solely in the initial recruitment of RNAP to promoter DNA (2). Thus, whereas both CRP and FNR function by interacting directly with αCTD, the consequences of these interactions appear to differ. This hypothesis is supported by the observation that FNR fails to recruit purified αCTD in electromobility shift assays (data not shown), unlike CRP, which binds α cooperatively (28). Interestingly, the surface of FNR that is responsible for contacting αCTD is much larger than the equivalent surface of CRP (2, 13). Further studies are needed to determine the individual contributions of the subregions of AR1 to the process of transcription activation.
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