Activation of the Escherichia coli Nitrate Reductase (narGHJI) Operon by NarL and Fnr Requires Integration Host Factor*

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Integration host factor protein (IHF) was shown to be required for Fnr- and NarL-dependent activation of the nitrate reductase (narGHJI) operon of Escherichia coli in response to nitrate availability and anaerobiosis. Using a narG-lacZ reporter fusion to evaluate narGHJI expression in vivo both the nitrate and anaerobic-dependent controls were severely impaired in a himA mutant compared with the wild type strain. IHF was also required for Fnr-independent anaerobic control of narGHJI expression. In vitro, purified IHF protein was shown to bind to a narG promoter fragment with an apparent dissociation value of 5 nM by use of a gel shift assay. DNase I footprinting studies revealed that IHF protects a 37-base pair region centered 125 base pairs 5' of the narG transcription site. These studies suggest that the IHF protein performs a DNA bending function at the narG promoter to allow nitrate-dependent activation by the NarL regulatory protein, and second, it enhances the Fnr-dependent expression from the narG promoter under anaerobic cell growth conditions. A model whereby three transcriptional activators, NarL, IHF, and Fnr, induce expression of a σ70-dependent promoter for the narGHJI operon is discussed.

The expression of the anaerobically inducible respiratory pathway genes in Escherichia coli for nitrate reductase (narGHJI), fumarate reductase (frdABCD), and dimethyl sulfoxide/thymelamine N-oxide reductase (dmsABC) requires the action of two transcription regulatory proteins called NarL and Fnr (1–6). Whereas Fnr functions as an anaerobic responsive sensor for the activation and repression of gene expression during anaerobic cell growth conditions (2, 7, 8), NarL functions as a nitrate- and molybdate-dependent response regulator of the NarX NarQ NarL two-component regulatory system (5, 6, 9–11). Upon activation by either the NarX or NarQ sensor-transmitter proteins, NarL binds to specific DNA sites to activate narGHJI operon expression. Distinct Fnr and NarL recognition sites have been proposed for the narGHJI promoter; the Fnr binding site is located immediately 5' of the −35 recognition domain for σ70 polymerase (7), while the NarL site is located well upstream of the transcription initiation site (i.e. 200 bp upstream of narGHJI 5' start (12)). For narGHJI operon expression, it was not evident how NarL and Fnr accomplish this task as each protein functions as an activator of transcription.

The expression of a number of genes in E. coli and other bacteria requires the presence of an accessory DNA binding protein called integration host factor (IHF) (13). This protein was originally identified due to its involvement in phage λ integration into the bacterial chromosome (14) and is required for proper expression of cellular proteins required for glutamate accumulation (glnH (15)), amino acid metabolism (16), osmotic adaptation (17), and utilization of xylose among others (18). IHF is a heterodimeric protein consisting of an α subunit encoded by himA and a β subunit encoded by himD (hip), and it can function as either a negative or positive transcription effecter (13). The protein has been shown to bind to specific DNA recognition sites and cause bending or looping of the DNA (19–21).

In this study we determined that the expression of the anaerobic electron transport pathway genes, narGHJI, required for nitrate utilization by the cell also requires the IHF protein. In addition we demonstrated in vitro IHF binding to the narGHJI regulatory region using purified IHF protein and located the IHF binding site 120 bp upstream of the narG promoter. These findings suggest a model for nitrate- and IHF-dependent activation of narGHJI operon expression.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Bacteriophages, and Plasmids—The E. coli strains and plages used in this paper are derived from MC4100 (F− araD139 Δ(argF-lac) U169 rpsL150 relA1 ilv5301 deoC1 ptsF25 rbsR) (22). The himA mutants, SD1 (himAΔ26 tetR), SD3 (himAΔ29 tetR), and SD29 (himAΔ29, ΔnarX) were constructed by P1-mediated transduction of the himAΔ26 (tetR) allele from K1299 (23) into MC4100, PC2 (ΔnarX (25)), and IS1 (ΔnarX (40)), respectively, and selection for tetramycin resistance (22). Transductants were screened for resistance to infection by phage Mu to confirm the presence of himA (14, 24). The himAΔ26 deletion strain used in this study is unable to synthesize IHF. Plasmid pLS65, which contains a 391-bp EcorI-BamHI narG promoter fragment, was used for gel shift and footprinting experiments. It was constructed by moving the 391-bp fragment from pFC50 (25) into pUC18. A narG-lacZ reporter fusion contained on APC60 (25) was used for the in vivo gene expression studies.

Cell Growth—For strain manipulations and maintenance, cells were grown in Luria broth or on solid media. For β-galactosidase assay, cells were grown in buffered L-broth (pH 7.0) (25). Where indicated, nitrate was added to an initial concentration of 40 mM. High aeration of cultures during aerobic growth was accomplished by shaking 15-ml culture volumes in 150-ml flasks (26).

β-Galactosidase Assays—β-Galactosidase assays were performed as previously described (4). β-Galactosidase values represent the average of at least three experiments with a variation of no more than 10% from the mean.

Galactosidase Assays—Gal retardation assays were performed as essentially described by Frenkli et al. (27). A 391-bp EcoRI-BamHI DNA fragment from pLS65, which contains the narG promoter region extending from nucleotide −236 to +155 relative to the transcription start, was isolated and end-labeled by filling in with Klenow DNA polymerase fragment and [γ32P]dATP (28). The labeled fragment (5 nM) was incubated with varying amounts of purified IHF (0.1–20.0

1 The abbreviations used are: bp, base pairs; IHF, integration host factor.
Role of IHF in Anaerobic narGHJI Gene Expression

Effect of a himA Mutation on in Vivo narGHJI Operon Expression—To determine if IHF protein has a physiological role in regulation of the nitrate respiratory operon, we tested the effect of a himA mutation on expression of a narG-lacZ reporter fusion. Cells were grown under aerobic and anaerobic conditions and either with or without nitrate, and β-galactosidase levels were measured (Table I). Relative to the isogenic parent (MC4100), expression of the narG-lacZ expression was severely impaired in a himA mutant under anaerobic conditions. When cells were grown in the absence of nitrate, narG-lacZ expression was reduced by about 4-fold in the himA mutant compared with the parent strain. In a fnr himA double mutant, narG-lacZ expression was significantly lower than observed for either the fnr or the himA single mutants (Table I), suggesting that IHF protein acts as a transcriptional enhancer of narG expression.

The nitrate-dependent activation of narG-lacZ expression was also altered in the himA mutant. A 1.6-fold nitrate induction seen in the wild type strain during aerobic growth was abolished whereas the 16-fold nitrate induction of narG-lacZ expression under anaerobic conditions was reduced to a 3.5-fold induction in the himA strain. Nitrate-dependent control was also abolished in the Δ(narXL) and the himA Δ(narXL) strains. Interestingly, this nitrate-dependent control was also abolished in a fnr mutant. Thus, nitrate induction of narG-lacZ expression appears to require the concerted action of three transcriptional activator proteins, NarL, Fnr, and IHF.

Location of the IHF Binding Site at the narG Promoter—DNase I footprinting experiments were performed to locate the IHF binding site(s) within the narG regulatory region (Fig. 1). A 391-bp DNA fragment containing the narG promoter with a 236-bp upstream region was used in the assay which is the same fragment used to construct the narG-lacZ reporter fusion employed in the in vivo expression studies (Table I). A 37-bp DNA sequence was protected by IHF when present at concentrations greater than 1 nM (Fig. 1). This IHF binding site is located at position -108 to -144 relative to the initiation site for narG transcription. At the DNA concentration used in this experiment, an apparent dissociation constant for IHF was estimated to be 5 nM based on a visual inspection of the gel shown in Fig. 1. When the concentration of IHF protein was increased to 50 nM in the footprinting assay, no additional protected regions were seen within the narG fragment.

Affinity of IHF for the narG Regulatory DNA—To better characterize IHF binding at the narG promoter, a DNA gel shift assay was performed using increasing amounts of highly purified IHF protein (Fig. 2). Compared with the mobility of the DNA fragment alone, a single DNA species with retarded mobility was observed upon addition of IHF protein to 20 nM. The amount of each retarded DNA species was determined by densitometry, and an apparent dissociation constant for IHF was calculated to be 5 nM. Hill plot analysis indicates a 1:1 stoichiometry of binding. However, if MgCl₂ and CaCl₂ were omitted from the DNA gel shift preincubation buffer, the DNA fragment was retarded at much lower concentrations of IHF (i.e. 0.5 nM), and when it was increased to 1 nM, a second gel shifted species was observed that migrated somewhat slower (data not shown). The DNA species exhibiting intermediate mobility bound IHF with a Kₜ value of 0.75 nM while the slower migrating DNA fragment bound IHF with a

RESULTS

Effect of a himA Mutation on in Vivo narGHJI Operon Expression—To determine if IHF protein has a physiological role in regulation of the nitrate respiratory operon, we tested the effect of a himA mutation on expression of a narG-lacZ reporter fusion. Cells were grown under aerobic and anaerobic conditions and either with or without nitrate, and β-galactosidase levels were measured (Table I). Relative to the isogenic parent (MC4100), expression of the narG-lacZ expression was severely impaired in a himA mutant under anaerobic conditions. When cells were grown in the absence of nitrate, narG-lacZ expression was reduced by about 4-fold in the himA mutant compared with the parent strain. In a fnr himA double mutant, narG-lacZ expression was significantly lower than observed for either the fnr or the himA single mutants (Table I), suggesting that IHF protein acts as a transcriptional enhancer of narG expression.

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### Table I

| Strain (genotype) | β-Galactosidase activity a |
|------------------|---------------------------|
|                  | Aerobic | Anaerobic |
| MC4100 (wild type) | 10      | 16       | 680      | 11,700 |
| SD1 (himA)        | 8       | 8        | 170      | 600    |
| PC22 (fnr)        | 11      | 18       | 110      | 123    |
| SD3 (himA, fnr)   | 10      | 8        | 22       | 22     |
| IS1 (narXL)       | 4       | 4        | 245      | 275    |
| SD20 (himA, narXL)| 5       | 4        | 143      | 118    |

a Cells were grown in buffered Luria Broth supplemented with 40 mM NaNO₃ where indicated.

b Units are given as nanomoles of ONPG hydrolyzed per min per mg of protein.

**Table I**

Effect of the himA gene product on expression of the anaerobic respiratory operon, narGHJI, as monitored by a narG-lacZ reporter fusion

**Fig. 1.** DNase I footprint of the IHF binding region upstream of the narG promoter. A 391-bp narG promoter DNA fragment was incubated with increasing amounts of IHF protein as indicated at the top of the figure (panel A) and digested with DNase I as described under "Experimental Procedures." The numbering of the DNA ladder is relative to the site of narG transcription. The IHF (40 nM) protected sequences shown in panel B are denoted by the region from nucleotides at position -108 to -144.
DNA that brings an upstream region nearby the RNA polymerase of the transcription start sites (15, 33, 34). Upon induction, IHF can bind to quite different recognition sites in the genome protected a 37-bp region at the promoter (Figs. 1 and 3). Inspection of the DNA sequence in this region centered -125 bp upstream of the transcription start (13). The dotted region below the IHF-protected 391-bp DNA fragment reveals a 13-bp element (TAAAACGTCCTTAA) that shows high similarity to an IHF consensus sequence proposed by Filutowicz and Inman (31) and Craig and Nash (19). Thus it appears that IHF binding, a conformational change is introduced in the DNA that brings an upstream region nearby the RNA polymerase binding site. For example, NifA-regulated promoters require IHF in combination with RNP to promote open complex formation and transcription initiation in Klebsiella pneumoniae (34). Thus, a mechanism for altering DNA topology of narG DNA is inferred.

DNA gel shift assays were performed using a narG promoter fragment that revealed a single high affinity IHF binding site (Fig. 2) consistent with the protected region revealed by the DNase I footprinting experiments (Fig. 1). Interestingly, if MgCl₂ and CaCl₂ were omitted from the gel shift reaction buffer, two distinct IHF retarded DNA species were observed that exhibited K₀ values of 0.75 and 5 nM. It is not known how MgCl₂ and/or CaCl₂ affect IHF to allow it to bind DNA with higher affinity or what the nature of the second retarded DNA species is that is observed in the gel shift assay when MgCl₂ and CaCl₂ are absent.

Based on the in vitro and in vivo narG studies presented herein, in addition to the demonstrated DNA bending properties of the IHF protein (20, 21, 35), a regulatory model for narGHJI operon expression is proposed (Fig. 4). NarL protein when bound at the upstream NarL site centered at position -190 is brought into the proximity of the narG promoter RNA polymerase complex by the DNA bending activity of IHF at positions -108 to -144. This proposal is based on IHF-dependent DNA bending, which in several instances has been reported to be about 140° (20, 21, 35). IHF-dependent DNA bending apparently brings relatively distant DNA sites (~200 bp) closer together in space to facilitate transcription initiation. This IHF-dependent conformational change at the narGHJI promoter is apparently required to both activate narGHJI gene expression in response to nitrate availability and to enhance narG expression under anaerobic conditions as neither process occurs optimally in a himA mutant. The positioning of NarL on the DNA is apparently critical to its function (12). When the NarL binding site was rotated either 4 bp closer or 4 bp further away from the narG promoter, nitrate activation was nearly abolished. Thus, IHF must also aid in the correct positioning of NarL to activate transcription. A residual nitrate-dependent activation of narG expression under anaerobic conditions was seen in the himA strain and may be due to limited DNA bending provided by the Fnr and/or NarL proteins. The case for Fnr protein-dependent DNA bending is based on its proposed structural similarity to the catabolite activator protein of E. coli (36). Catabolite activator protein also binds DNA at positions just 5' of the promoter and promotes transcription activation at least in part by its DNA bending ability (37, 38).
For full expression of the narGHJI operon, all three activator proteins (NarL, Fnr, and IHF) must apparently bind at their respective DNA recognition sites. A defect in any of the three proteins significantly impairs narG-lacZ transcription under inducing conditions (i.e., anaerobic growth in the presence of nitrate). It is neither clear nor implied by this model whether NarL, Fnr, IHF, and RNA polymerase physically interact to achieve optimal nitrate reductase operon expression or if they must simultaneously bind DNA. The IHF-protected region which is 125 bp upstream of the narGHJI promoter is approximately 90 bp from the Fnr consensus site and 60 bp from the predicted NarL site (Fig. 3). For none of the other genes regulated by IHF has a physical contact between IHF and an upstream regulator or RNA polymerase been demonstrated. Thus it is unlikely that IHF directly interacts with the NarL, Fnr, and RNA polymerase proteins. Goodman and Nash (39) reported that introduction of a DNA intrinsic bend could replace IHF function at the H2 locus of λ attP site in E. coli (39); thus the DNA conformational state induced by IHF serves as a regulatory element rather than a direct role for IHF protein itself. IHF presumably changes the narG DNA’s conformation in some fashion to bring NarL nearer to Fnr/RNP to facilitate nitrate-dependent transcription. However, the model shown in Fig. 4 does not clearly account for the enhanced narGHJI expression by IHF seen under anaerobic conditions when nitrate is absent from the culture medium. IHF is assumed to be present in the cell and bound to DNA under all conditions tested (Table I). Its role is presumably to enhance Fnr-NarL interactions at the narG promoter.

Interestingly, a 10-fold aerobic to anaerobic induction of narG-lacZ expression was still seen in a fnr mutant compared with a 68-fold effect seen in the wild type strain (Table I). Apparently an additional level of anaerobic control exists for narGHJI expression besides that provided by Fnr, an observation not previously noted. Interestingly, the 10-fold anaerobic induction effect seen in the fnr strain was nearly abolished in a fnr himA double mutant (Table I). IHF must somehow facilitate this Fnr-independent anaerobic control in addition to its role in enhancing Fnr and NarL interactions at the narGHJI promoter. It is also interesting to note that nitrate activation of narGHJI expression is severely impaired in a fnr mutant (Table I). Thus NarL’s ability to function as a transcriptional activator appears to depend on the presence of active Fnr under anaerobic conditions. A similar effect has been observed for nitrate activation of the narK gene by NarL and Fnr (40).

Repression of frdABCD and dmsABC operon expression during either aerobic or anaerobic growth conditions has been shown to be mediated by the NarL protein in response to nitrate (4, 5). The mechanism for this NarL repression process clearly differs from the ability of NarL to activate gene expression (2, 3). The NarL binding site for the narGHJII regulatory region is located well upstream of the narGHJII transcription start site (12) as is the case for the NarL site for activation of the narK gene of E. coli (40). In contrast, the NarL site for control of the frdABCD promoter overlaps the RNA polymerase binding site (2). For frdABCD and dmsABC operon expression, the NarL-dependent repression does not require IHF binding. Thus, a mechanism that accounts for nitrate activation of narGHJI expression by the three transcriptional activators, NarL, Fnr, and IHF, is clearly compatible with a quite different role for NarL as a negative regulator of other anaerobic respiratory pathways in E. coli, frdABCD, and dmsABC.

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