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Characterization of the DNA- and Metal-Binding Properties of *Vibrio anguillarum* Fur Reveals Conservation of a Structural Zn^{2+} Ion

EKATERINA E. ZHELEZNOVA,[1] JORGE H. CROSA,[2] AND RICHARD G. BRENNAN[1]*

Department of Biochemistry and Molecular Biology[1] and Department of Microbiology and Immunology,[2] Oregon Health Sciences University, Portland, Oregon 97201-3098

Received 4 February 2000/Accepted 4 August 2000

The ferric uptake regulator, Fur, represses iron uptake and siderophore biosynthetic genes under iron-replete conditions. Here we report in vitro solution studies on *Vibrio anguillarum* Fur binding to the consensus 19-bp *Escherichia coli* iron box in the presence of several divalent metals. We found that *V. anguillarum* Fur binds the iron box in the presence of Mn^{2+}, Co^{2+}, Cd^{2+}, and to a lesser extent Ni^{2+} but, unlike *E. coli* Fur, not in the presence of Zn^{2+}. We also found that *V. anguillarum* Fur contains a structural zinc ion that is necessary yet alone is insufficient for DNA binding.

Iron is essential for survival and virulence of bacterial pathogens; however, its concentration in host tissues is limited. To acquire iron, bacterial pathogens rely on iron uptake systems that consist of an iron siderophore (a small-molecule chelator) and membrane transport proteins, which import Fe^{3+}-siderophore complexes into the cell (7, 18). These systems are regulated by the DNA-binding protein Fur (ferric uptake regulator) in response to iron availability (3, 11, 20). When the intracellular concentration of iron increases above a certain level, Fur represses the transcription of genes encoding components of the membrane transport system as well as enzymes involved in siderophore biosynthesis.

To date, the most extensive biochemical studies have been limited to the *Escherichia coli* Fur protein, which in the presence of various divalent metals that act as corepressors binds a conserved 19-bp operator sequence, the iron box, which is located in the promoter region of iron uptake genes (4, 5, 9). Moreover, these studies have indicated that *E. coli* Fur possesses two metal ion-binding sites. One, the corepressor binding site, uses histidines and carboxylate ligands to coordinate binding of Fe^{2+} (and two of its functional mimics, Mn^{2+} and Co^{2+}) (1). This event promotes a conformational change that leads to DNA binding. The other cation-binding site is involved in protein structure and stability and binds Zn^{2+} with high affinity, using the thiols of Cys92 and Cys95 as two of the four coordinating ligands (2, 3, 14). These cysteines are essential for *E. coli* Fur activity both in vivo and in vitro (6).

Fur homologues have been identified in multiple bacteria, where they also regulate iron acquisition. In the fish pathogen *Vibrio anguillarum* strain 775, Fur represses production of a siderophore, anguibactin, and of Fe^{3+}-anguibactin transport proteins in response to abundant iron (21). *V. anguillarum* Fur is closely related to its counterparts from other *Vibrio* species, including *Vibrio cholerae* (93% sequence identity), *Vibrio vulnificus* (91%), and *Vibrio parahaemolyticus* (91%) (Fig. 1). Yet *V. anguillarum* Fur is less homologous to *E. coli* Fur (76% sequence identity), and the two proteins have different numbers of cysteines and histidines (Fig. 1). Therefore, to test whether *V. anguillarum* Fur is functionally distinct from *E. coli* Fur, we characterized the DNA- and divalent-cation-binding properties of *V. anguillarum* Fur. We also investigated the effect of chemical modification of cysteines on the ability of Fur to bind a consensus 19-bp iron box in a metal-dependent manner. Here, we report that Fur binds the iron box in the presence of Mn^{2+}, Co^{2+}, Cd^{2+}, and to a lesser extent Ni^{2+} but, unlike *E. coli* Fur, not in the presence of Zn^{2+}. In addition, *V. anguillarum* Fur contains a structural Zn^{2+} ion that appears to be required for DNA binding.

The *V. anguillarum fur* gene subcloned into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pT7-5 vector (21) was kindly provided by A. M. Wertheimer. *E. coli* BL21(DE3) cells transformed with this vector were induced with 1 mM IPTG. Fur, which contains nine histidines, binds tightly to nickel-nitrilotriacetic acid-agarose (Novagen) and is eluted in 1 M imidazole. Protein purity was assessed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. After dialysis against the storage buffer [0.1 M Tris HCl (pH 7.9), 0.1 M NaCl, and 0.1 to 0.2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl), in the presence or absence of 1 mM EDTA], the protein concentration was measured spectrophotometrically by using an extinction coefficient, ε_{280}, of 12,007 M^{-1} cm^{-1}, which was determined from amino acid hydrolysis.

The affinity of Fur for DNA was measured by fluorescence anisotropy (16). As it is performed in solution, this method provides a true equilibrium measurement of binding. All measurements were done using a Beacon fluorescence polarization instrument (Pan Vera) with excitation and emission wavelengths of 494 and 520 nm, respectively. The DNA used in this work is a 25-bp double-stranded oligonucleotide that encompasses the 19-bp iron box from *E. coli* (5). Each DNA duplex was prepared by annealing two complementary 25-mers, one of which contained a 5′ fluorescein label (Genosys and Oligos, Etc.). The sequence of the labeled nucleotide is 5′-GCAGATAATGATAATCATTATCGGA-3′. The inverted repeat of the iron box is underlined. All fluorescence anisotropy measurements were carried out at 25°C in 1 ml of binding buffer (100 mM HEPES with potassium salt [pH 7.5], 250 mM potassium glutamate, 150 mM sodium chloride, 10 mM magnesium acetate, and 5% glycerol) (15), with 1 μg of poly(dI · dC) per ml and 2 nM labeled DNA duplex. Fur was titrated into the mixture, and measurements were made after a 30-s equilibra-

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, L224, 3181 SW Sam Jackson Park Rd., Portland, OR 97201. Phone: (503) 494-4427. Fax: (503) 494-8393. E-mail: brennanr@ohsu.edu.

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V.anguillarum          ----------MSDNNQALKDAGLKVTLPRLKILEVLQQPECQHISAEELYKKLIDLGEE
V.cholerae             ----------MSDNNQALKDAGLKVTLPRLKILEVLQQPECQHISAEELYKKLIDLSEE
V.vulnificus           ----------MSDNNQALKDAGLKVTLPRLKILEVLQQPDCQHISAEDLYKKLIDLGEE
V.parahaemolyticus     ----------MSDNNQALKDAGLKVTLPRLKILEVLQQPDCQHISAEDLYKKLIDLGEE
E.coli                 ----------MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEE
K.pneumoniae           --------------------AGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEE
Y.pestis               ----------MTDNNKALKNAGLKVTLPRLKILEVLQNPACHHVSAEDLYKILIDIGEE
P.aeruginosa           ----------MVENSELRKAGLKVTLPRVKILQMLDSAEQRHMSAEDVYKALMEAGED
P.putida               ----------MVENSELRKAGLKVTLPRVKILQMLDSTEQRHMSAEECIKALMEAGED
R.leguminosarum        -------MTDVAKTLEELCTERGMRMTEQRRVIARILEDSE-DHPDVEELYRRSVKVDAK
B.japonicum            MTALKPSSASKASGIEARCAATGMRMTEQRRVIARVLAEAV-DHPDVEELYRRCVAVDDK
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V.anguillarum          IGLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVIEFSDEVIEQ
V.cholerae             IGLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVIEFSDDVIEQ
V.vulnificus           IGLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVIEFSDDIIEE
V.parahaemolyticus     IGLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVIEFSDDIIEE
E.coli                 IGLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVIEFSDDSIEA
K.pneumoniae           IGLATVYRVLNQFDDAGIVTRHNFESGKSVFELTQQHHHDHLICLDCGKEIEFSDDSIEL
Y.pestis               IGLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVIEFSNESIES
P.aeruginosa           VGLATVYRVLTQFEAAGLVVRHNFDGGHAVFELADSGHHDHMVCVDTGEVIEFMDAEIEK
P.putida               VGLATVYRVLTQFEAAGLVVRHNFDGGHAVFELADGGHHDHMVNVETSEVIEFMDAEIEK
R.leguminosarum        ISISTVYRTVKLFEDAGIIARHDFRDGGSRYETVPEEHHDHLIDLKTGTVIEFRSPEIEA
B.japonicum            ISISTVYRTVKLFEDAGIIERHDFREGRARYETMRDSHHDHLINLRDGKVIEFTSEEIEK
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V.anguillarum          RQREIAEQYNVQLTNHSLYLYGKCADG-SCKQNPNAHKSKR
V.cholerae             RQKEIAAKYNVQLTNHSLYLYGKCGSDGSCKDNPNAHKPKK
V.vulnificus           RQKEIAAAYNVQLTNHSLYLYGKCGDG-SCKGNPDAHKRKS
V.parahaemolyticus     RQREIAAKYNVTLTNHSLYLYGKCSDG-GCKENPDAHKPAK
E.coli                 RQREIAAKHGIRLTNHSLYLYGHCAEG-DCREDEHAHEGK-
K.pneumoniae           RQRGIASRHGIRLTNH------------------------
Y.pestis               LQREIAKQHGIKLTNHSLYLYGHCETG-NCREDESAHSKR-
P.aeruginosa           RQKEIVRERGFELVDHNLVLYVRKKK---------------
P.putida               RQREIVAEHGFELVDHNLVLYVRKKK---------------
R.leguminosarum        LQERIAREHGFRLVDHRLELYGVPLKKEDL-----------
B.japonicum            LQAEIARKLGYKLVDHRLELYCVPLDDDKPTS---------
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FIG. 1. Alignment of selected Fur sequences. Asterisks and colons indicate positions of identical and similar amino acids, respectively. Cysteines 92 and 95 of *E. coli* Fur and their counterparts in other Fur proteins are shown in bold.

tion. The DNA-binding assays were carried out initially in the presence or absence of Mn^{2+}, which has been shown to substitute effectively for the easily oxidized Fe^{2+} (9).

In the presence of Mn^{2+}, *V. anguillarum* Fur binds the consensus *E. coli* iron box with a K_d of 50 nM. In the absence of Mn^{2+}, *V. anguillarum* Fur does not bind the iron box and no changes in fluorescence anisotropy are observed, even at Fur concentrations greater than 2 μM (Table 1 and Fig. 2). Fur also binds its cognate DNA site with high affinity in the presence of Co^{2+} (K_d = 30 nM) or Cd^{2+} (K_d = 50 nM). In the presence of Ni^{2+}, *V. anguillarum* binds the iron box specifically, but with lower affinity (K_d = 230 nM). In the presence of Zn^{2+}, Fur does not bind the iron box, and the binding data are similar to those recorded in the absence of metal. By contrast, in the presence of Zn^{2+}, *E. coli* Fur binds the iron box specifically, albeit with a lower affinity (4, 9). These data indicate that, unlike *E. coli* Fur, *V. anguillarum* Fur cannot use Zn^{2+} as a corepressor ion.

To determine the concentration of Mn^{2+} that induces half-maximal binding, 2 nM iron box DNA in the presence of 500 nM *V. anguillarum* Fur was titrated with $MnSO_4$. The concentration of Mn^{2+} at which Fur achieves half-maximal DNA binding (apparent K_d) is 90 μM (data not shown). This affinity is very similar to that found for *E. coli* Fur (12) and suggests

TABLE 1. K_ds of *V. anguillarum* Fur for the consensus iron box operator

Fur	Metal[a]	K_d (nM)
Unmodified	Mn^{2+}	50 ± 2
Unmodified	Co^{2+}	30 ± 1
Unmodified	Cd^{2+}	50 ± 2
Unmodified	Ni^{2+}	230 ± 15
Unmodified	None	>2,000
Unmodified	Zn^{2+}	>2,000
DTNB modified	Mn^{2+}	500 ± 20

[a] The metal ion concentration was 1 mM in each binding experiment.

that the corepressor-binding sites of these two proteins are similar.

Recent studies have shown that *E. coli* Fur cysteine residues 92 and 95 are ligands for a structural Zn^{2+} ion (2, 14). The binding of this divalent cation is stabile and requires extreme treatment to effect its removal (2). Since these cysteines are conserved in *V. anguillarum* Fur, we wanted to determine whether *V. anguillarum* Fur, like its *E. coli* counterpart, contained a structural Zn^{2+} coordinated by cysteines. To do so, we measured the Zn^{2+} content of Fur protein using a Varian-Techtron flame atomic absorption spectrometer (λ = 213.9 nm). Fur that was dialyzed against storage buffer, with or without 1 mM EDTA, contained 1.4 ± 0.1 (mean ± standard deviation from three measurements per sample) mol of Zn^{2+} per mol of Fur. Interestingly, Fur dialyzed extensively against 50 mM EDTA also contained 1.4 ± 0.1 mol of Zn^{2+} per mol of protein. These amounts are different from those for the *E. coli* Fur protein, for which 0.5 to 0.8 (17) or 0.9 (2) mol of Zn^{2+} was found per mol of EDTA-treated protein, but 2.1 mol of Zn^{2+} was detected per mol of EDTA-free protein (2). The presence of only one Zn^{2+} ion per monomer in *V. anguillarum* Fur versus two Zn^{2+} ions in *E. coli* Fur correlates with the inability of *V. anguillarum* Fur to use Zn^{2+} as a corepressor and suggests that the corepressor-binding site of *V. anguillarum* cannot bind Zn^{2+}, whereas that of *E. coli* Fur can.

Next, we set out to determine the role of cysteines in Zn^{2+} coordination. To do so, we quantified the accessibility of the thiol side chains of the five cysteines of *V. anguillarum* as measured by their chemical modification by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (10, 19). If Cys92, Cys95, and possibly other cysteine residues were taking part in Zn^{2+} ion coordination, their chemical modification would eliminate Zn^{2+} from the protein sample. Our results showed that in the presence of 1 mM EDTA under both nondenaturing and denaturing conditions, 5.2 mol of cysteine per mol of *V. anguillarum* Fur was modified, which indicates that all five cysteine residues were accessible to DTNB. After DTNB labeling, Fur was dialyzed against its storage buffer, and subsequent Zn^{2+} analysis indicated the presence of 0.15 ± 0.05 mol of Zn^{2+} per mol of DTNB-labeled Fur protein. Concomitant with this dramatic decrease in Zn^{2+} content is a 10-fold-lower affinity of

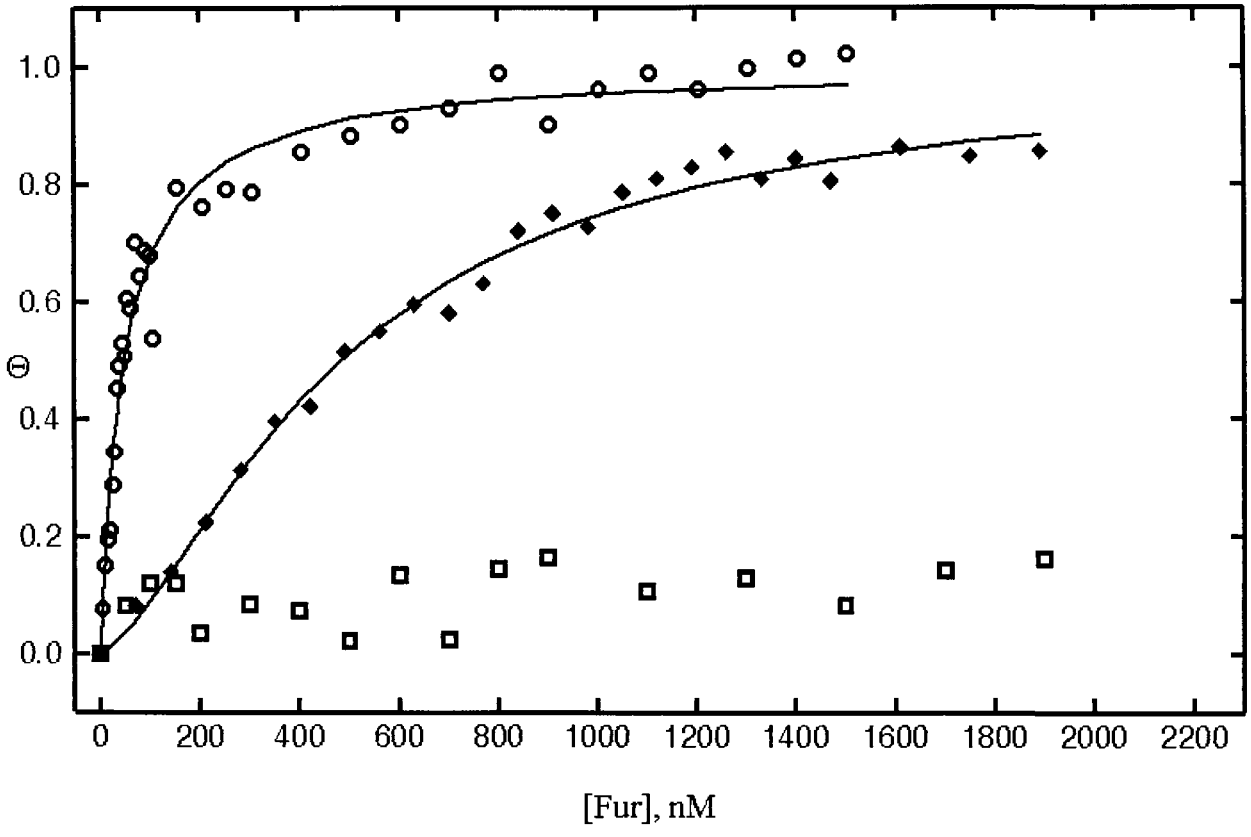

FIG. 2. Binding isotherms of unmodified Fur to the consensus iron box in the presence (○) and absence (□) of Mn^{2+} and binding of DTNB-modified Fur in the presence of Mn^{2+} (◆). Binding data were analyzed and fitted by nonlinear least-squares regression (Sigma Plot) to the following equation describing a cooperative binding model: $\theta = (A - A_0)/(A_{max} - A_0) = [P]^h/(K_d + [P]^h)$, where θ is the fractional saturation of the operator with Fur, A is anisotropy, A_{max} is maximum anisotropy, A_0 is initial anisotropy, [P] is the total protein concentration, and h is the Hill or cooperativity coefficient, which was in the range of 2.0 to 2.5. The graph shows θ as a function of total Fur concentration.

DTNB-labeled Fur for the iron box (K_d = 500 nM) in the presence of 1 mM Mn^{2+} ion.

The results of our thiol modification experiments indicate that a Zn^{2+} ion likely serves as the structural cation in *V. anguillarum* Fur, because modification of all five cysteines eliminates Zn^{2+} and significantly diminishes DNA binding. These results are similar to those reported for the *E. coli* Fur, which binds Zn^{2+} at a structural site (14) using cysteines 92 and 95, and this Zn^{2+} stabilizes the protein (2). Specifically, mutagenesis of these two cysteines in *E. coli* Fur (6) as well as their chemical modification (8) results in diminished DNA binding. Further experiments are necessary to determine whether cysteines 92 and 95 are responsible for Zn^{2+} binding in *V. anguillarum* Fur. While these two cysteines are conserved between the *E. coli* and *V. anguillarum* Fur proteins, they are replaced in the Fur proteins of several other bacteria, such as *Pseudomonas putida* and *Bradyrhizobium japonicum* (Fig. 1) (13). Hence, it is unclear whether the structural Zn^{2+} found in *E. coli* and *V. anguillarum* Fur is universally used in Fur proteins from other bacteria and whether the presence of cysteines at positions 92 and 95 is indicative of the structural Zn^{2+}-binding site. Although necessary, this Zn^{2+} is not sufficient for high-affinity DNA binding by *V. anguillarum* Fur, which also requires the presence of a divalent corepressor ion, such as Mn^{2+}. On the contrary, *E. coli* Fur was recently reported to bind its operator with high affinity in the presence of only its structural Zn^{2+} (2). However, this finding will require additional investigation. Perhaps a side-by-side study of the two Fur proteins would clarify the basis of their different metal ion-binding properties.

In summary, our results reveal that the closely related *E. coli* and *V. anguillarum* Fur proteins possess comparable divalent cation- and DNA-binding characteristics, albeit with significant differences. These findings illustrate the overall conservation of function and metal specificity of Fur proteins from these different gram-negative bacteria and thus expand our understanding of these essential regulatory proteins.

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