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The Ferric Uptake Regulation (Fur) Repressor Is a Zinc Metalloprotein†

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ABSTRACT: The Fur protein regulates the expression of a wide variety of iron-responsive genes; however, the interaction of this repressor with its cognate metal ion remains controversial. The iron-bound form of Fur has proved difficult to obtain, and conflicting results have been published using Mn(II) as a probe for in vitro DNA-binding studies. We report here that the purified protein contains tightly bound zinc and propose that Zn(II) is bound to the protein in vivo. Upon purification, Fur retains ca. 2.1 mol of Zn(II)/mol of Fur monomer (Zn_2Fur). One zinc is easily removed by treatment of Zn_2Fur with zinc chelating agents, resulting in Zn_1Fur with ca. 0.9 mol of Zn(II)/mol of protein. The remaining zinc in Zn_1Fur can only be removed under denaturing conditions to yield apo-Fur with ca. 0.1 mol of Zn(II)/mol of protein. Our results suggest that many literature descriptions of purified Fur protein do not correspond to the apo-protein, but to Zn_1Fur or Zn_2Fur. Dissociation constants (K_d) of protein−DNA complexes are ca. 20 nM for both Zn_2Fur and Zn_1Fur as determined by electrophoretic mobility shift assays and DNase I footprinting assays. The two metalated forms, however, show qualitative differences in the footprinting assays while apo-Fur does not bind specifically to the operator. The existence of these Zn(II) binding sites in Fur may resolve some discrepancies in the literature and have implications concerning Zur, a Fur homologue in *E. coli* that regulates zinc-responsive genes.

The ferric uptake regulatory (Fur)[1] protein mediates iron-responsive regulation of a variety of *Escherichia coli* genes, including those involved in iron uptake (*1, 2*). It has also been shown to regulate genes not directly related to iron transport, such as those encoding hemolysin (*3*), Shiga-like toxin (*4*), colicin I receptors (*5*), and Mn- and Fe-containing superoxide dismutases (*6*). The promoters for iron uptake genes are negatively regulated by Fur; low iron concentrations in the media result in derepression, while high iron concentrations result in Fur-dependent repression (*7, 8*). Based on evidence from in vitro transcription/translation coupled assays, the Fur metalloregulatory protein is proposed to act as a classical repressor in which Fe(II) is required as a cofactor for DNA binding (*9*). Fur exhibits DNA binding in the presence of other divalent metal ions such as Mn(II), Co(II), Cu(II), and Cd(II) in addition to Fe(II) in vitro (*9, 10*). It was proposed that Fur does not bind DNA in the absence of divalent metal ions. Several mechanistic issues remain to be established. It is not clear how Fur differentiates among various divalent metals, how metal binding alters DNA-binding activity, or how DNA-binding activity controls transcription of the target genes.

The expressed version of Fur, a 147 amino acid protein with a molecular mass of 17 kilodaltons (kDa), contains 12 His and 4 Cys residues (*11, 12*). These residues have been identified as likely candidates for metal coordination; however, the metal ion binding site in Fur is controversial. Results from paramagnetic NMR studies implicate coordination of histidines and carboxylate to a Mn(II) ion, which has been assumed to be bound in the putative Fe(II) site of Fur (*13*). Furthermore, from spin-labeling experiments it was suggested that the four reduced cysteines are not involved in Mn(II) binding. In contrast, however, electronic absorption spectra of Cd(II)-substituted Fur and the phenotype of Cys→Ser Fur mutants support a role for cysteine involvement in metal binding (*14, 15*). Enhanced rates of Fur proteolysis in the presence of excess metal were previously observed, which suggests that metal binding may affect protein conformation (*14*). It was also reported that effects on the DNA affinity of Fur are only observed in the presence of excess metal (*9, 10, 16*).

The molecular basis of metal ion recognition and the nature of the metal-induced conformational changes in the protein, which in turn affect gene expression, are fundamental questions in this system. To elucidate the mechanism, the properties of well-defined metal−Fur protein complexes must be correlated with the biochemical behavior of the protein. Here we show through metal-binding assays that Fur, as

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[1] Abbreviations: BisTris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; DTNB, 5, 5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fur, ferric uptake regulation protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICP-AE, inductively coupled plasma atomic emission; IEF, isoelectric focusing; LB, Luria Bertani broth; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMPS, *p*-(hydroxymercuri)phenylsulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Zn-IDA, zinc iminodiacetate; 2-ME, 2-mercaptoethanol; TBE, Tris−borate/EDTA electrophoresis buffer.

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isolated by different methods, is not an apo-protein, but instead contains zinc. Three forms of the protein have been prepared and characterized: Zn_2Fur, Zn_1Fur, and apo-Fur. In both electrophoretic mobility shift and DNase I protection experiments, we show that zinc binding to Fur correlates with increased DNA-binding affinity. Other studies have indicated that Fur can bind zinc (*9*, *10*, *17*, *18*); however, the functional roles of zinc have not been explored. Given the evidence presented here on the robust nature of Zn_1Fur in vitro and in vivo, it is likely that many of the previous in vitro studies of Fur were conducted unknowingly with zinc-containing protein. It is difficult to ascertain from the literature which form of the protein, Zn_2Fur or Zn_1Fur, was examined since zinc analyses were generally not carried out. Interpretations of metal effects observed in DNase I footprinting experiments with Fur (*10*), HinfI protection, and in vitro coupled transcription−translation experiments (*9*) may need to be reconsidered.

Our results indicate that Zn(II) binding to Fur is more than an artifact of the purification procedures, and that one of these zinc-bound forms of Fur is a "resting state" of the protein in the cell. These studies of zinc-binding sites in Fur are consistent with the recent discovery of a Fur homologue in *E. coli*, Zur, which appears to act as a zinc sensor (*19*).

MATERIALS AND METHODS

Materials. Zinc iminodiacetate (Zn-IDA) agarose resin was obtained from Pierce Chemical Co. Centricon-10 concentrators and YM-10 filters from the Amicon Division of W. R. Grace & Co. were used for ultrafiltration. Chelex-100 (100−200 mesh) resin and a Bradford protein assay kit with bovine gamma globulin (IgG) and bovine serum albumin (BSA) standards were purchased from Bio Rad Laboratories. Pharmacia TSK-DEAE ion-exchange resin, isoelectric focusing (IEF) gels, and broad-range calibration kits were used. Radioactive nucleotides were obtained from Amersham. All other reagents and materials of the highest grade were obtained commercially and used without further purification.

The pARC306FUR plasmid is pREC-driven and contains the same *fur* coding sequence as pMON2064 (*12*). The host strain, *E. coli* JM101, was used from laboratory stocks.

Protein Production and Purification. Method 1. JM101 cells were transformed with the overexpression vector pARC306FUR by standard molecular cloning methods (*20*). JM101/pARC306FUR cells originating from a single colony were grown in LB media. At an optical density between 0.4 and 0.6, the cells were induced with nalidixic acid. Noninduced cells were grown to approximately the same optical density as the induced cells. Prior to lysis, both sets of cells were washed in 20 mM Tris-HCl, pH 8.0, with 50 mM EDTA and pelleted, and the supernatant was decanted. Crude extracts were prepared by resuspension of the various cell pellets in equal volumes of 50 mM Tris-HCl, pH 8.0, buffer with 1.25 mM PMSF. Following lysis by sonication, the cell debris was removed by centrifugation. Streptomycin sulfate (1% w/v) was added to the supernatant, and the resulting precipitate was then removed. The supernatant was brought to 80% saturation in ammonium sulfate to precipitate the protein. The crude protein was redissolved and then purified on a Zn-IDA column (1.6 cm × 15 cm) as described (*12*). Purified Fur was exchanged into Hepes buffer (20 mM Hepes, pH 7.0, 0.1 M NaCl, 2 mM DTT, 5% glycerol) by gel filtration on a Sephadex G25 column (2.6 cm × 30 cm) in the absence of EDTA.

Method 2. Crude extract was prepared as above. The ammonium sulfate precipitate was first diluted and dialyzed in 20 mM Tris, pH 8.0. Protein was loaded on a TSK-DEAE anion exchange column (1.6 cm × 50 cm). The column was washed with 200 mL of 20 mM Tris-HCl, pH 8.0, and Fur was eluted along a 0−0.2 M NaCl gradient.

Protein Analysis. The purity of the isolated protein was examined on a 15% SDS−PAGE gel (30:0.8) stained with 0.025% Coomassie Blue G in 10% acetic acid. Protein bands in the dried gel were analyzed by densitometry on a Pharmacia LKB 2222-020 UltraScan XL laser densitometer. The Pharmacia GelScan XL software program was used to integrate absorbance peaks arising from the protein bands.

Protein Determination. Protein concentration was determined by several methods. Purified Fur samples were submitted in duplicate to the Northwestern University Biotechnology Facility for total amino acid hydrolysis to quantitate the precise amount of protein. Portions of the sample were lyophilized and hydrolyzed in 6 M HCl at 155 °C for 1 h. The hydrolysate was derivatized and dissolved in 1 mL of buffer. The dissolved samples were analyzed by HPLC for residue content by comparison to amino acid standards. Since the amino acid sequence of expressed Fur is known, the protein concentration could be deduced from the residue concentration (*11*, *12*). The same protein sample was also assayed by the Bradford method (*21*) with IgG as the standard and by its absorbance measurement at 276 nm in 6 M Gdn-HCl.

A procedure for estimating the molar absorption coefficient of proteins based on the number of Trp, Tyr, and Cys residues was also employed (*22*). This theoretical method is most consistent for normal globular proteins with an error of 5−10%. Since Fur has no Trp, four Tyr, and four Cys residues as deduced from the known amino acid sequence (*11*), molar absorption coefficients of $\epsilon_{276} = 6380\ M^{-1}\ cm^{-1}$ and $\epsilon_{280} = 5600\ M^{-1}\ cm^{-1}$ were calculated.

Metal Analysis. Metal content in protein and buffer solutions was measured on a Thermo Jarrell Ash AtomScan 25 ICP-AE spectrometer in the Analytical Service Facility at Northwestern University. Average metal concentrations and standard deviations were determined from four measurements of the same sample.

Dialyses at Various pHs. Zn_2Fur or Zn_1Fur was dialyzed in 20 mM buffer (acetate, citrate, BisTris-Cl, MOPS, Hepes, or Tris-HCl) ranging from pH 4.8 to 8.5 with 0.15 M NaCl and 1 mM DTT. One milliliter samples (50−100 μM) were dialyzed in 250−500 mL of buffer containing 0.5−1.0 g of Chelex resin outside the bag. The buffer was changed 3 times. Protein was then retrieved from the dialysis bags and assayed. The final dialysis buffer was also analyzed for zinc.

Zinc Removal. A method for removing 1 mol of zinc from Zn_2Fur was developed by incubating a 100 μM protein sample in 50−500 mM EDTA at pH 8.0 for 1 h to overnight or by dialyzing a 1 mL portion against 500 mL of 50 mM EDTA at pH 8.0. Subsequently, the sample was loaded on a Sephadex G-25 (1.6 cm × 25 cm) column in Hepes buffer.

Complete removal of zinc from Zn_1Fur to prepare apo-protein requires either dialysis at low pH or treatment with urea and a strong chelating agent. In the first procedure, 1

mL of 50−100 μM Fur was dialyzed against 1 L of 50 mM sodium acetate (NaOAc) buffer, pH 5.0, with 0.1 M NaCl, 2 mM 2-ME, and 1 g of Chelex-100 resin for 1 day with one buffer change. The Fur sample was collected and analyzed for protein and zinc.

Apo-Fur can also be prepared by incubating 200 μM of zinc-containing Fur in 1 mL of 7 M urea, 50 mM EDTA, 100 mM DTT at pH 8.0. Following incubation, the protein was diluted in 10 mL of Hepes buffer with 10 mM DTT and concentrated to less than 1 mL under positive N_2 pressure in an Amicon ultrafiltration apparatus equipped with a YM-10 filter. This diafiltration step continued with five more dilution−concentration cycles. Protein was collected and stored in an acid-washed tube. The Fur concentrate was then assayed to contain ca. 0.1 mol of zinc/mol of protein.

Preparation of Metal-Free Buffers and Labware. Special precautions were taken to eliminate trace Zn(II) when working with apo-Fur. Glassware and plasticware were acid-washed and rinsed with deionized water and Milli-Q water. Buffers were treated with Chelex-100 resin as described (*23*).

Zinc Reconstitution. Apo-Fur (50−125 μM) was incubated with 1−4 equiv of zinc chloride ($ZnCl_2$) and 8−14 equiv of DTT or 2-ME in 10 mM Hepes buffer. After 1−4 h, the mixture was loaded on a Sephadex G25 column and eluted with Hepes buffer with 1 mM DTT to remove unbound metal. Fractions were then analyzed for protein and zinc content. Zn_1Fur (25−50 μM) was incubated with 5−10 equiv of $ZnCl_2$ and 20 equiv of DTT overnight at 4 °C. Samples were then gel filtered in 0.1 M sodium phosphate (NaP_i) buffer, pH 7.0, with 0.5 M NaCl and 5% glycerol. Fractions were then collected and analyzed.

β-Galactosidase Assays. The bacterial strain used in these assays, KP1022, was supplied by Kathleen Postle. KP1022 is a W3110 lysogen containing the *tonB* promoter cloned into the expression vector λRS205, resulting in *tonB*−*lacZ* operon fusion. TonB is a membrane protein that is proposed to transport Fe(III)−siderophore complexes across the outer membrane. *TonB* transcription is repressed in the presence of $FeCl_3$, and repression is mediated by the *fur* gene (*24*). β-Galactosidase assays with KP1022 were conducted in chelexed M9 minimal media which contains M9 salts, 0.2% glucose, 4 μg/mL thiamin, 1 mM $MgCl_2$, and 0.2% casamino acids (Difco Laboratories, Detroit, MI). The medium was supplemented with varying levels of freshly dissolved $ZnCl_2$ or $FeCl_3$ to determine the effects of these metals on *tonB* transcription. Nitrilotriacetate (NTA) was also added to the growth medium to loosely chelate free Fe(III), thereby preventing precipitation of ferric hydroxide complexes which interfere in OD_{600} measurements. β-Galactosidase activity was assayed as described by Miller (*25*).

Gel Filtration. Apparent molecular weights (M_r) of various protein samples were determined by high-resolution gel filtration on a Pharmacia Superose 12 column (1.6 cm × 30 cm) at 4 °C. Fur samples (≈100 nmol) or gel filtration standards were diluted in 200 μL of chelexed buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT) and then run in the same buffer. Each sample also contained 0.5 mg/mL blue dextran (MW 2×10^6) as a void reference. The Superose 12 column was run at a flow rate of 0.25 mL/min, the absorbance was monitored at 280 nm, and absorbance peaks were integrated on Pharmacia's fast protein liquid chromatography (FPLC) system. The standard curve was plotted as the elution volume (V_e)/void volume (V_o) versus the log MW of standards, and the linear fit was calculated by using the Cricket Graph software program.

Cysteine Determination. Free cysteine concentration in the protein was assayed with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent, according to a method described in the literature (*26*, *27*). Steps were taken to ensure that the solutions remained anaerobic throughout the assay (*28*). The DTNB reaction was also conducted in air with Fur samples prepared in 20 mM Hepes, pH 8.0, 1 mM EDTA, and 2% SDS, which are the buffer conditions previously described in the literature (*29*).

Isoelectric Focusing (IEF). The isoelectric points (p*I*) of Zn_2Fur, Zn_1Fur, and apo-Fur were determined by isoelectric focusing (IEF) on Pharmacia's PhastSystem. Fur samples (50 μM) were loaded on a PhastGel IEF 5−8 along with broad calibration standards using a 6/4 comb. Separation conditions and Coomassie Blue staining methods prescribed by Pharmacia were used. The protein migrating distance from the cathode (R_f) was measured and compared with the standards.

UV−Vis and Circular Dichroism Spectra. Various Fur samples were desalted on a Sephadex G25 column in 100 mM NaP_i, pH 7.0. Protein concentrations were determined in triplicate by the Bradford method with IgG standards. Metal concentrations were determined from four measurements by ICP-AE spectroscopy. Optical measurements were carried out on a Hewlett-Packard 8452 UV−Vis diode array spectrophotometer. UV−Vis spectra were recorded from 200 to 800 nm in a 1 cm quartz cuvette. CD spectra of protein (≈2 μM) were measured in a 0.1 cm path length quartz cell on a Jasco 500C spectrophotometer. Data were collected from 400 to 190 nm at 0.2 nm increments.

Construction of Plasmid pBIB and Gel Mobility Shift Assays. Two "iron box" oligonucleotides consisting of the consensus Fur-binding site (*10*) were synthesized at the Northwestern Biotechnology Facility: 5′-GAT AAT GAT AAT CAT TAT CG-3′ and 5′-GAT CCG ATA ATG ATT ATC ATT ATC TGC A-3′. Equal amounts of the two oligonucleotides (20 pmol) were annealed to form double-stranded (ds) DNA with *Pst*I and *Bam*HI sticky ends. The dsDNA was inserted into pBluescript SK ± vector (Stratagene) at the *Pst*I and *Bam*HI sites.

The constructed plasmid pBIB was cut with *Hind*III and *Pvu*II restriction enzymes to yield a 287 bp fragment used as a DNA template in electrophoretic mobility shift assays. The fragment containing the "iron box" was then labeled with [α-^{32}P]dGTP and [α-^{32}P]dCTP (Amersham) by the Klenow fragment of DNA polymerase and isolated from a 5% (30:0.8) polyacrylamide gel. DNA template concentrations were measured by absorption at 260 nm by using a molar extinction coefficient of 13 000 M^{-1} cm^{-1} per nucleotide pair. $[DNA^*]_{max}$ is the value assuming 100% recovery from the gel.

Electrophoretic mobility shift conditions have been reported previously (*16*). Assay conditions were modified to eliminate $MnCl_2$ from all buffers and were performed at pH 7.0. The labeled DNA probe and various concentrations of Fur protein were incubated in binding buffer consisting of 20 mM BisTris (pH 7.0), 2.5 μg/mL sonicated salmon sperm DNA, 5% (w/v) glycerol, 100 μg/mL bovine serum albumin, 1 mM $MgCl_2$, 40 mM KCl, with or without different metal

salts at the designated concentrations. The reaction was carried out at room temperature for 30 min and then loaded on a 5% (30:0.8) nondenaturing gel containing 20 mM BisTris buffer (pH 7.0) and electrophoresed at 180 V for 2 h.

After drying, the gel was applied to a Molecular Dynamics phosphorimager. The DNA−protein complexes were detected by ^{32}P radioactive emissions. The signals were digitized and analyzed by the ImageQuant v3.0 program (Molecular Dynamics) with the local background redefined as zero. Data are presented by plotting the fraction of DNA bound versus the log of Fur concentration using the Igor software program (WaveMetrics, Inc., Lake Oswego, OR). Since the concentration of Fur is about 100-fold greater than that of DNA under the experimental conditions, data can be plotted on a sigmoidal graph. The apparent K_d for the individual experiments is the concentration of Fur at the half-maximal binding (*30*). The best estimate of K_d was obtained by averaging the values of at least three sets of experiments.

DNase I Footprinting of the tonB Promoter. The plasmid pRZ540 with the complete *tonB* gene was supplied by Kathleen Postle (*31*). A fragment comprising 160 bp downstream and 230 bp upstream of the transcription start site was amplified using PCR. This 400 bp fragment was then blunt-end-ligated into *Sma*I-digested pUC19 (New England BioLabs). The resulting plasmid (pUC19TonB) was digested with *Eco*RI and labeled with [α-^{32}P]dATP and dTTP using the Klenow fragment of DNA Polymerase (New England BioLabs). The linearized plasmid was then digested with *Bam*HI to produce a 400 bp fragment labeled at one end. This fragment was purified on a 5% (30:0.8) polyacrylamide gel. The end-labeled DNA fragment (0.2−1.0 nM) and various amounts of either Zn_2Fur, Zn_1Fur, or apo-Fur were incubated for 30 min at 37 °C in 200 μL of footprinting buffer [10 mM BisTris (pH 7.0), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM KCl, 100 μg/mL bovine serum albumin, and 2.5 μg/mL sonicated salmon sperm DNA]. Ten microliters of 50 nM DNase I was then added to the reaction mixture and allowed to incubate for 1 min at 37 °C. The reaction was terminated with the addition of 700 μL of DNase I stop solution (560 mM NH_4OAc, 30 μg/mL yeast tRNA, 86% EtOH), vortexed, and then precipitated on dry ice for >30 min. The pellets were washed with 200 μL of 70% EtOH, dried under vacuum, and then resuspended in 6 μL of loading buffer (0.5× TBE, 80% formamide, 0.05% xylene cyanol FF, 0.05% bromophenol blue). The samples were denatured for 10 min at 95 °C, quenched on ice, and then loaded onto a sequencing gel (7% polyacryamide, 7 M urea, 1.2× TBE). The same DNA fragment was also cleaved in a Maxam−Gilbert guanine-specific reaction (*20*) for use as a DNA sequence ladder.

The dried gel was digitized in the same manner as the gel shift experiments described above using a Molecular Dynamics phosphorimager. The fractional protection of the Fur binding site at each protein concentration was then obtained using a quantitative footprint titration technique (*32*). The fractional protection was plotted versus the log of the Fur concentration in the same manner as the gel shift data to obtain the apparent K_d.

RESULTS

Molar Absorption Coefficient Determination. The absolute Fur concentration is critical for establishing metal−protein stoichiometry. Results from total amino acid hydrolysis and precise protein quantitation indicate that the Bradford method, using IgG as a standard, overestimates the Fur concentration by a factor of 2.8. To convert from IgG to Fur, the concentration, expressed in terms of milligrams of IgG per milliliter, is multiplied by 0.35. We calculate a molar absorption coefficient of $\epsilon_{276} \approx 6600 \pm 50$ M^{-1} cm^{-1} for purified Zn_2Fur in 100 mM NaP_i, pH 7.0, buffer when protein concentration is determined by the Bradford method using our correction factor. In a previous report, a 1 mg/mL solution of Fur assayed by the Bradford method had an A_{275} of 0.4, which corresponds to a molar absorption coefficient of $\epsilon_{275} \approx 6720$ (*12*). Both experimental values are consistent with the calculated value of 6380 M^{-1} cm^{-1}. We also find that the Bradford method overestimates protein concentration by 1.2 times using BSA as the standard.

Metal−Protein Stoichiometry. Fur purified using the Zn-IDA column was measured to be 99% pure by densitometry (see Materials and Methods) and contained less than 0.05 mol of Fe/mol of Fur monomer. Instead, it contained 2.1 ± 0.5 zinc atoms per Fur monomer, which we designate Zn_2Fur. This form of the protein is stable to gel filtration on a Sephadex G25 column in 0.1 M NaCl. We originally set out to completely remove all metal atoms from Zn_2Fur by incubating or dialyzing the protein in the presence of strong zinc chelating agents; however, 0.9 ± 0.1 mol of zinc/mol of Fur remained in the protein. This form is denoted as Zn_1Fur. Incubation or dialysis of Zn_2Fur in excess EDTA at pH 8.0 and subsequent gel filtration consistently yielded Zn_1Fur. Other chelating agents such as dipicolinate or nitrilotriacetate (NTA) yielded similar results.

Error is expressed in terms of standard deviations that are typically calculated from three or more experiments in which each sample is measured in triplicate for metal and protein. Propagation of error for the metal:protein ratios can be as great as 20%, by assuming 2.5% pipetting errors in the Bradford assays and in the amino acid hydrolysis determination and 5% error in the ICP measurements.

Crude Extract Studies. Since zinc binding to Fur may arise from purification by zinc affinity chromatography, we tested the possibility that Fur acquires Zn(II) before the purification step. The induced JM101/pARC306Fur cells contained more protein than in the uninduced cells (Figure 1), and zinc levels in the former were also elevated. The iron concentration in the induced cells, however, did not vary greatly from that in the control cells. Increasing levels of zinc appear to correspond with increasing levels of Fur protein during overproduction. This result indicates that Fur accumulates zinc inside the cell prior to purification.[2]

Alternative Purification Method. Another method of purification that avoided the use of a Zn−IDA column was explored to minimize Fur exposure to adventitious zinc sources. Fur protein purified on a TSK-DEAE ion-exchange column was eluted at ≈0.16 M NaCl along the salt gradient.

[2] To roughly estimate the proportion of zinc bound to Fur in the crude extracts, the concentrations of metal and protein in the uninduced control cells were subtracted from those in the induced cells. By assuming that Fur is the primary component after induction, the approximate proportion of zinc-to-Fur in the induced cells was calculated to be 0.5 [Althaus, E. W. (1995) Thesis, Northwestern University].

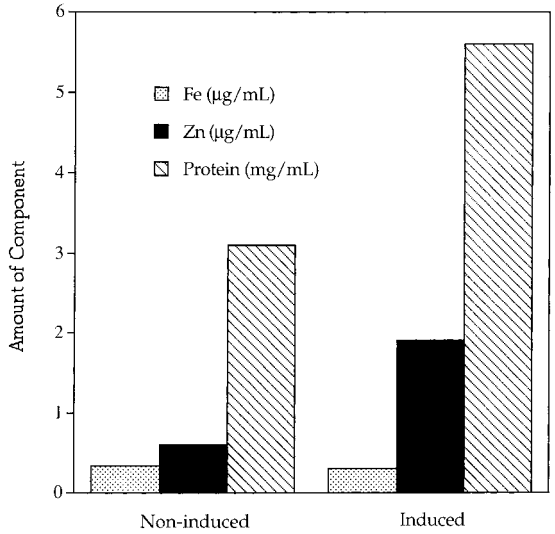

FIGURE 1: Metal and protein analysis of crude extracts from induced and control JM101/pARC306Fur cells. JM101/pARC306Fur cells were grown in LB to an optical density between 0.4 and 0.6 after which one set was induced with nalidixic acid. Induced and control cells were grown to the same density, and extracts were prepared as described under Materials and Methods. Protein concentration was determined using the Bradford method with IgG as the standard.

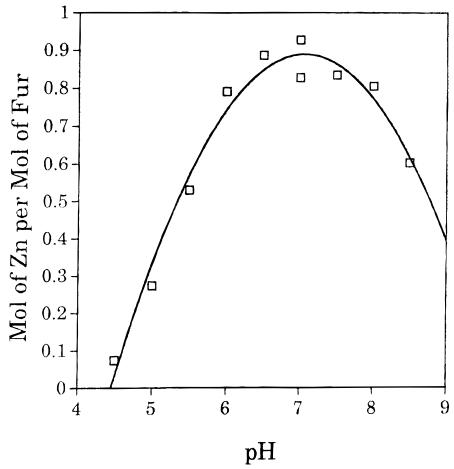

FIGURE 2: Zinc to Fur monomer ratio versus dialysis buffer pH. Zn_2Fur is dialyzed in buffer at various pHs with Chelex-100 resin outside the dialysis bag as described under Materials and Methods. Fur samples were recovered and analyzed for protein and zinc.

The final product contained 0.7 mol of Zn/mol of protein and showed 70% purity on an SDS−PAGE gel.

Effect of pH on Zn Removal. Dialysis of Zn_2Fur at various pH values in the presence of Chelex-100 resin resulted in the removal of 1 mol of zinc/mol of Fur. With decreasing pH of the dialysis buffer, zinc content decreases (Figure 2). At pH 4.8, virtually all Zn(II) in Fur was removed. At lower pH, an insoluble precipitate formed. This could be reduced to some extent by increasing the ionic strength of the dialysis buffer with 0.1 M NaCl. The zinc-to-Fur proportion reaches a maximum around pH 7.0 and then decreases at pH values greater than 7.0, with 0.6 mol of Zn/mol of protein remaining in the Fur sample dialyzed at pH 8.5.

Preparation of Apo-Fur. Apo-Fur could be prepared by dialyzing 1 mL of 50−100 μM purified Fur at 4 °C in various buffers (pH 5.0) in the presence of Chelex-100 resin. The final product contained 0.1 mol of Zn(II)/mol Fur. Although

Table 1: Various Zinc Removal Treatments of Fur[a]

[EDTA] (mM)	[DTT] (mM)	[urea] (M)	mol of Zn/mol of Fur[b]
50	100	7	0.1 ± 0.1
50	1	−	0.9 ± 0.1
50	1	4	0.1 ± 0.1
50	100	−	0.9 ± 0.1
50	−	7	0.2 ± 0.1
−	100	7	0.9 ± 0.1

[a] Fur protein at 100 μM concentration is incubated under the various conditions listed above at pH 8.0 overnight at 4 °C and then diafiltered in metal-free Hepes buffer at pH 7.0 containing 10 mM DTT. The protein concentration of each sample is measured in triplicate by the Bradford method. Zinc concentration is determined 4 times by ICP-AE spectroscopy. [b] Averages and standard deviations of zinc-to-Fur proportions are determined from 3−12 separate experiments.

Table 2: Summary of Various Treatments of the Fur Protein

treatment	mol of Zn/ mol of Fur[a]	designation
none	2.1 ± 0.5	Zn_2Fur
50 mM-0.5 M EDTA, pH 8.0	0.9 ± 0.1	Zn_1Fur
7 M urea, 0.5 M EDTA, 100 mM DTT	0.1 ± 0.1	apo-Fur
apo-Fur + 1−4 equiv of Zn(II) + DTT[b]	1.2 ± 0.1	Zn_1Fur
Zn_1Fur + 5−10 equiv of Zn(II) + DTT[b]	1.7 ± 0.8	Zn_2Fur

[a] Averages and standard deviations calculated from 3−12 experiments. [b] See Materials and Methods for specific amounts.

nearly all zinc is eliminated from Fur, there are limitations to this procedure. When the volume of reagents was scaled up, losses of Fur by precipitation during the dialysis lead us to explore other methods.

Incubation of 100 μM Zn_2Fur with 50−500 mM EDTA, pH 8.0 at 4 °C overnight was not sufficient to completely remove zinc from Fur. Fur retains 0.9 ± 0.1 mol of Zn/mol of Fur under these conditions. Introduction of a denaturation/renaturation step, however, resulted in successful preparations of large amounts of apo-protein. When 100 μM Fur samples were incubated in 7 M urea, 0.5 M EDTA, and 100 mM DTT at pH 8.0 and subsequently washed with metal-free buffer, 0.1 ± 0.1 mol of Zn/mol of Fur remained (Table 1). By using this method, zinc is removed with greater efficiency than the low-pH dialysis method, and the risk of thiol oxidation is reduced since fresh DTT is used in the wash step as the protein is concentrated under N_2 gas.

In control experiments summarized in Table 1, both urea and EDTA are necessary for consistent zinc removal. Incubations of Zn_2Fur in DTT-containing buffers with EDTA or urea alone gave 0.9 ± 0.1 mol of Zn(II) bound/mol of Fur.

Zinc Reconstitution. Apo-Fur was reconstituted with 1−4 equiv of Zn(II) in the presence of thiols in 20 mM Tris, pH 7.0, or Hepes buffer to produce Zn_1Fur. The apo-protein appears to have a high affinity for zinc as it readily binds trace zinc from untreated buffer over a 24 h period to form Zn_1Fur. Additional zinc binding to Zn_1Fur requires incubation with excess Zn(II) and thiols. Following gel filtration on a Sephadex G25 column in the absence of EDTA, approximately two zinc atoms are bound to Fur (Table 2).

In Vivo Transcriptional Regulation by Fur. As shown in Figure 3, *tonB* transcriptional activity, indicated by β-galactosidase units, is repressed approximately 2-fold by increasing levels of $FeCl_3$. On the other hand, over a wide range of $ZnCl_2$ concentrations, *tonB* transcriptional activity

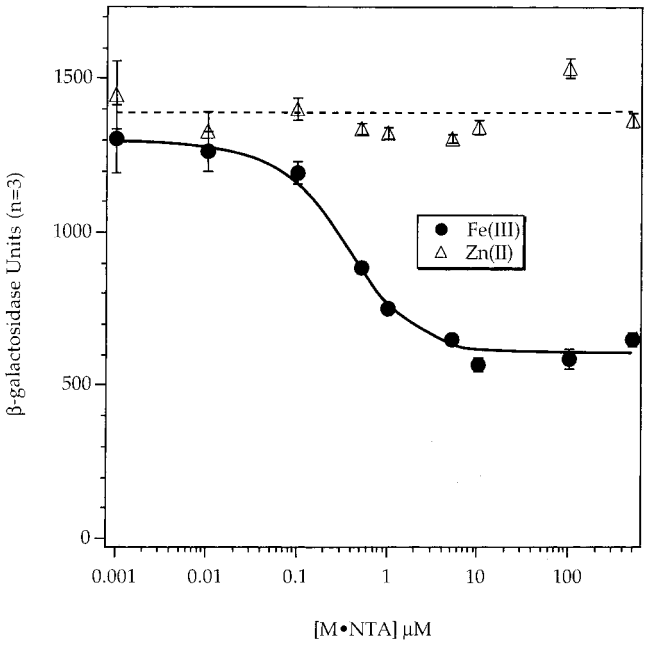

FIGURE 3: In vivo β-galactosidase assays of transcriptional activity of the *tonB* gene. The *E. coli* strain KP1022 was grown in chelexed M9 minimal media supplemented with the indicated amount of metal chloride. All experiments were determined in triplicate, with the error bars indicating one standard deviation both above and below the average values.

Table 3: Summary of Biochemical and Biophysical Characterization of Zn_2Fur, Zn_1Fur, and Apo-Fur

protein	mol of RSH[a]	M_r[b]	pI[c]
Zn_2Fur	2.6 ± 0.1	48000[d]	5.4
Zn_1Fur	2.8 ± 0.2	45000	5.4
apo-Fur	2.7 ± 0.1	32000	6.0

[a] Moles of RSH per mole of Fur determined by titration with DTNB under nondenaturing conditions. [b] Apparent molecular weight (M_r) determined by gel filtration chromatography on a Superose 12 column (1.6 cm × 30 cm) at 4 °C in 20 mM Tris-HCl, pH 8.0, with 100 mM NaCl buffer and 1 mM DTT. [c] Isoelectric points (pI) were determined by isoelectric focusing on a Pharmacia PhastGel system using an IEF 5−8 gel. The uncertainty is ±0.1. [d] One mole of zinc is removed from Zn_2Fur during the Superose 12 column run.

remains constant. These results indicate that *tonB* transcription, which has been shown to be Fur-regulated, is not responsive to zinc levels, suggesting that Fur is not a zinc sensor. This result is in accord with the absence of zinc effects on other Fur-regulated genes including *sodA*, which encodes Mn-containing superoxide dismutase (*33*), and *iucC*, a gene involved in the biosynthesis of the siderophore aerobactin (*9*). The bound zinc must play some other role in the protein's function.

Cysteine Determination. Approximately three cysteine residues (Table 3) are accessible to DTNB in each form of the protein—Zn_2Fur, Zn_1Fur, and apo-Fur—under nondenaturing conditions. When subjected to denaturing conditions (2% SDS) in the presence of EDTA, zinc was removed from the protein, as shown by gel chromatography and elemental analysis, and the apparent thiol content of all three forms increased to 4−5 cysteine residues per monomer. These values greater than 4 and the value of 3.4 titratable cysteines in the literature reports (*12*, *29*) may be due to the presence of SDS in the assay, which is known to perturb the molar absorption coefficient of TNB^- (*27*).

Isoelectric Focusing. Since the zinc content in Fur varies according to treatment, we examined the pI values of Zn_2Fur, Zn_1Fur, and apo-Fur to see if metal binding confers a change in the overall charge of the protein. As shown in Table 3, both Zn_2Fur and Zn_1Fur exhibit a pI = 5.4, whereas apo-Fur has a pI = 6.0. A p$I \approx$ 5.85 was reported previously for Fur purified on a Zn−IDA column; however, the presence of Zn(II) in the protein was not determined, and thus it is not clear which form of the protein was analyzed (*12*). Apo-Fur exhibits a slightly higher pI value than its Zn(II)-containing counterparts; however, the effect is subtle. This slight increase in the overall charge of the apo-protein suggests a possible difference between protein conformations in the apo form and the metalated forms.

UV−Vis and Circular Dichroism Spectroscopy. There are no obvious differences in the UV−visible absorption and circular dichroism (CD) spectra of Zn_2Fur, Zn_1Fur, and apo-Fur. CD spectra for all three Fur forms exhibit similar α-helical features with comparable molecular ellipticity (data not shown).

Molecular Weight Determination of Zn_2Fur, Zn_1Fur, and Apo-Fur. The observed apparent molecular weight of the apo-Fur sample, $M_r \approx$ 32 000, in the 50−500 μM concentration range is in agreement with Fur existing as a dimer under these conditions (Table 3). The Zn_2Fur sample loses 1 equiv of zinc during gel filtration on a Superose 12 column, and the subsequent product migrates corresponding to a $M_r \approx$ 48 000. We note that the loss of zinc from this form of the protein is dependent on the chromatography media as both equivalents of zinc are retained by the protein when Sephadex G25 is employed. Samples of Zn_1Fur migrate at approximately $M_r \approx$ 45 000. The gel filtration results for Zn_2Fur and Zn_1Fur samples are consistent either with a change in shape of the protein relative to apo-Fur or with a trimeric state, based on the molecular weight of monomeric Fur (MW 16 800). An apparent molecular weight of $M_r \approx$ 41 000 for the wild-type Fur protein by native polyacrylamide gel electrophoresis was previously reported (*15*); however, the Zn(II) content of that protein was not noted.

Electrophoretic Mobility Shift Assays. High levels of $MnCl_2$ (100−150 μM) are typically used in published DNase I footprinting and electrophoretic mobility shift experiments involving Fur (*10*, *16*). Mn(II) is presumed to saturate the putative Fe(II) binding site of Fur. Under these conditions, complications may arise from nonspecific binding of Mn(II) or from redox chemistry of Mn(II) during gel electrophoresis. We find it unnecessary to use $MnCl_2$ in electrophoretic mobility shift assays or footprinting assays in order to detect binding activity. Under these conditions, optimal gel shifts occurred at pH 7.0 rather than at pH 7.5. The protein−DNA dissociation constant (K_d) for Zn_2Fur, Zn_1Fur, and apo-Fur was determined by electrophoretic mobility shift assays. A typical assay for Zn_2Fur is shown in Figure 4. In gel shift experiments, Zn_2Fur and Zn_1Fur demonstrate similar affinities for DNA with dissociation constants of K_d = 18 ± 7 nM and 19 ± 5 nM, respectively (Table 4). The dissociation constant for apo-Fur, K_d = 48 ± 10 nM, is markedly greater than that of either Zn_2Fur or Zn_1Fur. Apo-Fur reconstituted with 1 mol of Zn(II) regains DNA-binding activity with a K_d comparable to that of Zn_1Fur.

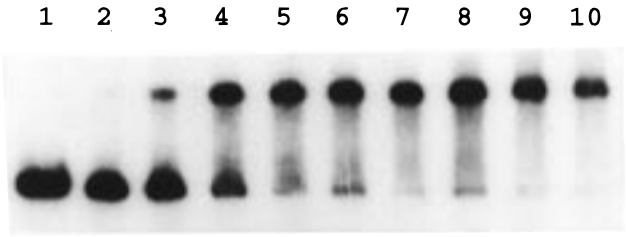

FIGURE 4: Electrophoretic mobility shift assay of Zn_2Fur. DNA concentrations were ≈10 pM. Protein concentrations varied between the lanes as follows: lane 1, 0 nM; 2, 1 nM; 3, 5 nM; 4, 10 nM; 5, 20 nM; 6, 30 nM; 7, 40 nM; 8, 50 nM; 9, 100 nM; 10, 200 nM.

Table 4: Summary of Fur−DNA Dissociation Constants (K_d)

	K_d (nM)[a]	
sample	gel shifts	DNase I footprinting
Zn_2Fur	18 ± 7	20 ± 5
Zn_1Fur	19 ± 5	21 ± 7
apo-Fur	48 ± 10	no binding

[a] K_d = ([Fur][DNA])/[Fur·DNA]. In these experiments, [Fur] ≫ [DNA]. K_d = [Fur] for half-maximal DNA binding ([DNA] = [Fur·DNA]).

DNase I Footprinting. The DNase I footprinting confirmed the binding constants of both Zn_1Fur and Zn_2Fur obtained from gel shifts; however, the footprinting patterns of each form were markedly different (Figure 5). Both forms bind with equal affinity to the Fur box located between −10 and −35 in the *tonB* promoter (*34*); however, the footprint of Zn_2Fur extends from +50 to −95 at Fur concentrations >300 nM. Once the concentration reaches 1 μM, the entire length of the DNA appears to be completely protected from cleavage. Throughout this same concentration range, the Zn_1Fur footprint does not extend beyond the promoter Fur box.

In contrast to Zn_2Fur and Zn_1Fur, apo-Fur did not show a clear footprinting pattern. Typically, one or two lanes in the titration experiment exhibited a footprint between −10 and −35, but the concentrations in which this occurred varied from one experiment to another (three different experiments were performed). Random zinc contamination from the binding buffer, Eppendorf tubes, or the pipet tips may account for the sporadic binding. The binding buffers for both footprinting and gel shift assays were found to contain less than 30 nM zinc by ICP-AE spectroscopy. The binding seen in gel shift experiments may be due to zinc contamination from other sources, such as the running buffer, the gel matrix, and even the glass plates, which are not found in the footprinting assay.

DISCUSSION

Given the abundance of histidine residues (8%) in Fur, some binding of Zn(II) to this protein is not surprising. It is unexpected, however, that Fur has such a high Zn(II) content after purification under conditions that typically remove adventitiously bound metal ions. Several new lines of evidence lead us to propose that the native Fur protein is a zinc metalloprotein. First, the protein as isolated by literature methods is not the apo form as widely assumed, but is either Zn_2Fur or Zn_1Fur, depending on the final steps. Although EDTA effectively removes 1 mol of zinc from Zn_2Fur under physiological conditions, EDTA alone is ineffective in removing the second zinc ion from Zn_1Fur. Second, apo-Fur can only be obtained at low pH or in the presence of a denaturant to promote metal release. Third, binding of 1 mol of Zn(II)/mol of apo-Fur is facile. In fact, apo-Fur will acquire zinc from standard buffers that have not been treated with Chelex. Metal analyses of crude extracts and protein purified on a TSK-DEAE column suggest that a substantial amount of zinc is bound to Fur without exposure to the standard column used in Fur purification, Zn-IDA. Finally, upon overexpression of Fur in *E. coli*, the cells accumulate significant quantities of zinc. Under these conditions, the amount of Fur may have exceeded the supply of available iron; however, the recalcitrant zinc binding demonstrated here leads us to propose that the native form of Fur found in cells grown with normal levels of iron in the media is Zn_1Fur or Zn_2Fur.

In previous work, some precautions were taken to remove excess metal ions that might remain after purification on a Zn-IDA column, including desalting Fur in EDTA-containing buffer or dialysis against 50 mM EDTA (*12*, *13*, *15*). Although not explicitly stated, it was assumed that the resulting form of Fur was apo-protein. Hence, specific binding of Zn(II) to Fur was not experimentally tested in previous studies. Since none of the typical treatments of Fur described in the literature will lead to extraction of the tightly bound zinc, it should be assumed that samples of Fur protein in published studies also contained tightly bound zinc. In some cases described below, the interpretations may need to be reevaluated in light of these observations.

Zinc and Protein Stability. Purified Fur binds two nonequivalent zinc atoms: one tightly bound and one weakly bound. A similar model is proposed for RNA polymerase (*35*, *36*). One function of zinc binding to Fur appears to involve stabilization of protein architecture. Based on NMR studies, Saito and others propose that Fur consists of a rigid core (*13*). Our evidence suggests that this core may be in part stabilized by a zinc ion, which can only be removed under denaturing conditions (acid or urea). Upon total zinc removal, the conformation and DNA affinity of Fur seem to change as demonstrated by a decrease in the apparent molecular weight (M_r), a slight increase in the isoelectric point (p*I*), and a decrease in DNA-binding affinity. Furthermore, reconstitution of apo-Fur with 1 mol of Zn(II) restores the DNA affinity of the protein. The DNA affinity of apo-Fur in gel shift experiments further decreases to zero within 4 days at 4 °C with no DTT or 2-ME present, whereas the activity of Zn_2Fur is retained over the same time period (data not shown). The three forms of the protein were also run on an SDS−PAGE gel after 7 days at 20 °C with no DTT or 2-ME (data not shown). All three proteins when freshly thawed from the freezer showed one band at 17 kDa. However, after 7 days, apo-Fur was ∼80% degraded into smaller fragments ranging from 5 to 16 kDa, while both Zn_2Fur and Zn_1Fur remained ∼50% intact with only one proteolytic fragment at 16 kDa. The enhanced susceptibility to trace protease contamination supports the notion that apo-Fur is unstable without zinc.

The instability of apo-Fur may be due to various factors. Fur may be unfolded without a zinc to stabilize its active conformation, rendering it more vulnerable to protease impurities. Cysteine residues, which are either coordinated to the zinc center or protected in the interior of the protein

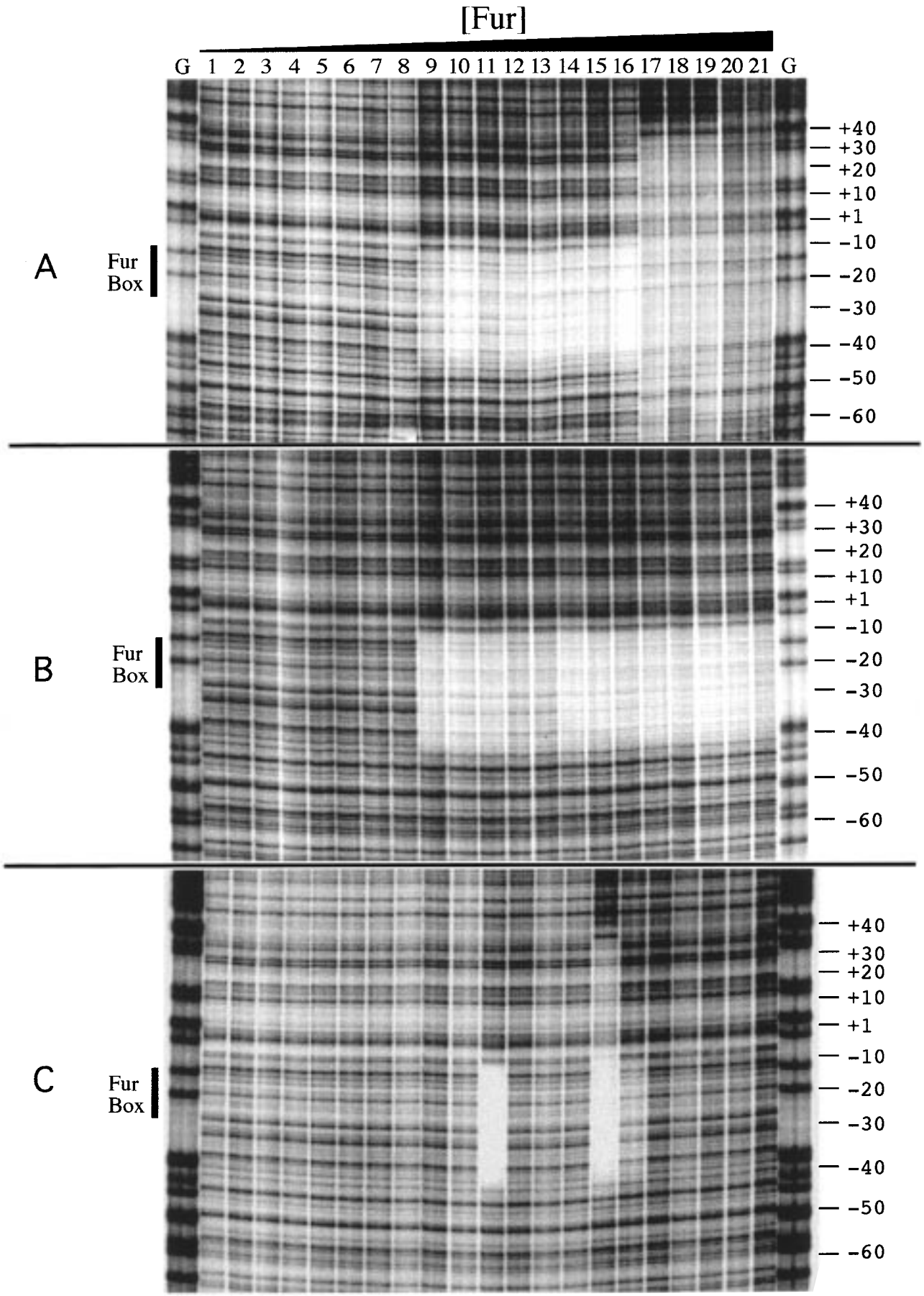

FIGURE 5: DNase I footprinting assays of (A) Zn_2Fur, (B) Zn_1Fur, and (C) apo-Fur. Lanes 1−21 correspond to the following increasing protein concentrations (nM): 0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 30, 40, 60, 80, 100, 200, 300, 400, 600, 800, 1000, 1500, 2000. The lanes labeled G are guanine-specific DNA sequence ladders. The Fur binding site (Fur box) is indicated by the filled rectangle on the left side of each gel.

when one zinc ion is bound, may be more susceptible to oxidation in the apo-protein.

Metal-Binding Sites. The issue of what type of side chains coordinate to the metal center in Fur has been controversial. Site-directed mutagenesis of Cys92 and Cys95 to serine residues causes an approximate 100-fold decrease in DNA affinity of the Fur mutants compared to wild-type Fur (*15*). The reduced DNA affinity of the Fur mutants was assumed to arise from disruption of an iron-binding site. In NMR studies using Mn(II) as a paramagnetic broadening reagent and a probe for the iron-binding site, Saito and others concluded that histidines and carboxylates, but not cysteines, are coordinated to Mn(II), which was presumed to occupy the iron-binding site (*13*). The discrepancy between these results can be resolved by the two-site model for metal binding described above. Our observations that Fur binds two zinc atoms per monomer suggest that the NMR and mutagenesis results are providing information on two separate sites.

The coordination environment in a zinc-Fur sample, designated by Jacquamet et al. as apo-Fur, has recently been studied by X-ray absorption spectroscopy (*17*). To avoid confusion, we suggest that the term 'apo-Fur' be applied to protein stripped of all transition metals. The zinc in these samples is bound in a tetrahedral environment with two sulfur donor ligands and two N/O donor ligands. These results are consistent with the data reported here; however, our experiments raise the possibility that the EXAFS results represent the average of two distinct sites.

Gel filtration, isoelectric focusing, circular dichroism, and UV−Vis absorption spectroscopy provide little evidence that clearly differentiates Zn_2Fur from Zn_1Fur. The only apparent difference between the two forms is their interaction with DNA at high protein concentrations. The extension of the footprint beyond the promoter Fur box seems to be induced by binding of the second zinc to Fur (Figure 5). Polymerization of Fur protein on DNA has also been observed by microscopy (*37*); however, the zinc content of the protein was not measured in that study. A report of an enhanced rate of proteolysis in the presence of excess metal ions also indicates that metal binding induces a conformational change in Fur (*14*). Since it is likely that the 'apo-Fur' sample in those studies was actually the Zn_1Fur form, those observations may indicate the effects of metal binding at a second site.

Preliminary results indicate that zinc competes to some extent with the putative corepressor for Fur, Fe(II). Incubation of apo-Fur with 12 equiv of Fe(II) under inert atmosphere and removal of unbound metal result in both iron and zinc binding to Fur, although no Zn(II) metal ions were added intentionally. Apo-Fur thus appears to bind trace Zn(II) in preference to excess Fe(II). Furthermore, we find that iron does not displace zinc in Zn_1Fur; rather 1 equiv of iron binds to the form Fe(II),ZnFur. Additional biochemical work with Fe(II),ZnFur under strictly anaerobic conditions is necessary to resolve the effect of Fe(II) on Fur's DNA-binding activity. Binding of the second zinc may take place in the putative Fe(II)-binding site, since Fe(II) will displace one Zn(II) in Zn_2Fur.

The recent discovery of the Zur protein, a Fur homologue that regulates zinc uptake in *E. coli* (*19*), has implications for our proposal that Fur is a zinc metalloprotein. The 19 kDa Zur protein is 25% identical to Fur and has several mechanistic and evolutionary similarities to the Fur protein. Furthermore, sequence similarities in the His and Cys clusters implicate some conservation of the Zn-binding sites in the two proteins. We tested a role for Fur in cellular responses to changes in the zinc concentration in the growth media. Elevated zinc concentrations clearly do not induce repression of the Fur-responsive *tonB* gene, indicating that Fur is not functioning as a zinc sensor.

Zinc Effects on Fur/DNA Interactions. Our DNA footprinting experiments with Zn_2Fur, Zn_1Fur, and apo-Fur reveal moderate DNA-binding activity for Zn_2Fur and Zn_1Fur but not for apo-Fur. Since it is easier to control zinc levels in the footprinting than in the gel shift experiments, we find the footprinting results more reliable. The apo-form does not show consistent DNA binding in the footprinting experiment, leading us to conclude that this form does not exhibit specific DNA binding, at least up to the micromolar Fur concentration range. We surmise that the weak binding of apo-Fur seen in gel shift experiments was caused by zinc contamination that converted apo-Fur to Zn_1Fur or Zn_2Fur.

Metal Effects on Fur−DNA Footprints. The effects of divalent cations on Fur/DNA interactions in vitro in other studies are puzzling in light of the clear in vivo iron-specific response of Fur-regulated genes. Both *Hin*fI restriction digest protection by Fur (*9*) and DNase I footprinting protection of the aerobactin operator region (*10*) lead those authors to suggest that Fur exhibits little divalent cation specificity. In those experiments, Fur (60 nM) was shown to protect the Fur box of the aerobactin operator from DNase I cleavage after addition of 150 μM Mn(II), Co(II), Cd(II), or Cu(II) to the reaction mixture. In addition, de Lorenzo et al. show that 75−100 μM Zn(II) induces very weak DNase I protection by Fur and also partially inhibits the effects of 150 μM Mn(II) in the same experiment. The authors concluded that Zn(II) does not elicit the same effects on Fur binding to DNA and repressor activity as the other divalent metals. Since no analytical data specifying the zinc content in Fur were provided, it is unclear which form of the protein was studied. A key difference between the de Lorenzo experiments and those shown in Figure 5 is the nature of the metal−protein complex. In the former case, the protein is mixed with excess metal and EDTA. This presumably allows saturation of available metal-binding sites, but it can also lead to nonspecific effects on protein−DNA interactions. The experiments in Figure 5 were conducted with metal−protein complexes of fixed stoichiometry. The absence of excess metal or EDTA allows the direct evaluation of metal−protein interaction on DNA binding.

We have repeated some experiments of de Lorenzo et al. using Zn_1Fur and obtained similar results (data not shown); however, we suggest an alternative to their explanation that may reconcile some of the published discrepancies. We suggest that moderate (i.e., micromolar) EDTA concentrations can directly interfere with protein−DNA interactions in this case. We find that Zn_1Fur binds to DNA in the presence of 200 nM EDTA, but the DNA-binding affinity of Zn_1Fur is inhibited at least 10-fold at higher EDTA concentrations (i.e., 100 μM). Binding activity is increased when the EDTA-containing mixture is supplemented with 100 μM metal (*38*), suggesting that an anionic EDTA molecule may interact with Fur and disrupt protein−DNA

contacts. Since EDTA is not capable of removing the Zn(II) from Zn_1Fur at this concentration (see Table 2), we conclude that it is not interfering in binding by pulling zinc out of Fur. Also, we suggest that EDTA is not inhibiting Fur/DNA binding by chelating metals that may contaminate the footprinting buffer. If there were significant levels of metals contaminating the buffer, we would expect apo-Fur to be active in the footprinting assays without EDTA present. The apparent lack of divalent cation specificity noted by de Lorenzo et al. can be reconciled by a model in which Fur and the added metal ions compete for binding to EDTA. Once EDTA is saturated with other divalent metals, its inhibitory effects diminish and DNA protection by the Zn-Fur form can be observed. In this scenario, the added divalent ions are not directly affecting Zn-Fur, but are rather removing an inhibitor that interferes with DNA binding.

The presence of zinc in Fur may offer some explanation for unusual observations mentioned in other studies as well. For example, in vitro transcription−translation coupled assays with extracts of the bacterial Fur overproducer demonstrated repression of a *lacZ* fusion to the aerobactin operon without additional metal (*9*). Repression was attributed to adventitious metal binding in the assay and was eliminated with 10−35 μM EDTA. It was proposed that EDTA removed adventitious metals to form apo-Fur, which does not bind DNA. Alternatively, we propose that the apo-Fur in these studies was Zn_2Fur or Zn_1Fur, which was able to bind DNA and repress transcription. It is likely that one zinc was removed from Zn_2Fur to form Zn_1Fur by the addition of EDTA; however, the differences seen with and without EDTA are best attributed to micromolar levels of EDTA interfering with Fur−DNA binding, which then appeared as derepression.

The EDTA competition hypothesis begins to address the issue of why in vitro DNA binding of Fur can apparently be induced by a variety of metals other than Fe(II) when the primary function of this purported Fe(II) receptor is to regulate iron levels inside the cell. Other models also need to be considered. There are several precedents in the literature showing that Zn(II) may be substituted for an Fe(II) site, or vice versa. Such is the case with overproduction of the iron protein rubredoxin in *E. coli*; up to 70% of the protein is isolated in the zinc-bound form (*39*). The Zn(II) sites in metallothionein can be substituted with Fe(II) (*40*). The erythroid transcription factor GATA-1 binds specifically to DNA as a Zn(II) or Fe(II) complex (*41*). The unresolved problem in the Fur case is how the correct signal for a change in intracellular iron is distinguished from a change in intracellular zinc concentration. This remains an open question.

Models for Metal-Dependent Fur Regulation. The classical Fur repressor model with Fe(II) as cofactor needs to be reevaluated in light of our results. There are at least two different metal-binding sites in Fur, but only one of the two sites is a readily exchangeable metal center. In a scenario where one metal site is inert and merely serves a structural role, the classical repressor model could still be valid. The possibility of more than one site in Fur has been considered by others to explain multiple metal binding in equilibrium dialysis experiments with excess divalent metal (*14, 42*).

Our results modify the model that apo-Fur is the resting state of the protein which binds Fe(II) to form the active repressor (*9*). We propose that Zn_1Fur (or Zn_2Fur) is the resting state of the protein in vivo. Furthermore, we suggest that the affinity of either of these forms of Fur for DNA is not sufficient to lead to significant occupancy of the Fur box in vivo for at least one Fur-responsive promoter (*tonB*). For example, we have shown that Zn−Fur complexes bind with moderate affinity (K_d = 20 nM) to the Fur box in the *tonB* promoter in vitro without the addition of Fe(II). This site in the *tonB* promoter is not occupied by Fur in in vivo footprinting assays unless there are high iron levels in the growth media (*34*). Thus, for the *tonB* promoter, it is likely that Zn_1Fur or Zn_2Fur corresponds to the derepressed state which does not have sufficient affinity to saturate the Fur operator until the signal of high iron concentration in the media is transmitted to the Fur protein.

Another possibility which cannot be ruled out is that repression is induced after Fe(II) binding to the Zn_1Fur/DNA complex. Major changes in protein−DNA affinity are not necessarily required for transcriptional switching, as seen with the metalloregulatory protein MerR, which regulates Hg(II) detoxification genes. Apo-MerR and Hg(II)-MerR bind DNA with a 3-fold difference, but only the latter activates transcription (*30*).

Finally, as the simple repression/depression model for Fur is further tested, it should be noted that Fur may not sense concentrations of intracellular iron by directly binding the metal ion. There is no evidence to refute the possibility that high-affinity DNA binding by Fur requires interaction with a second component as observed in the two-component iron regulatory system of the ferric citrate uptake (*fec*) genes in *E. coli* (*43*). Efforts to address these alternatives and to study the activity of Fe(II),ZnFur are underway.

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