Metal Binding Characteristics and Role of Iron Oxidation in the Ferric Uptake Regulator from *Escherichia coli*†

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Received April 25, 2005; Revised Manuscript Received July 23, 2005

ABSTRACT: The ferric uptake regulator is a metal-dependent transcription repressor that is activated by divalent transition metal cations. Fe(II) is believed to be the primary functional metal in vivo; however, the ability of other divalent cations to activate Fur brings into question the true physiological metal. Furthermore, the role of different oxidation states of iron in activating Fur has not been determined. Comparison of the affinity of different metals with intracellular metal concentrations would suggest which metals activate Fur in vivo; however, no accurate determinations of the affinity of Fur for metals have been reported. In this study, methods for reconstituting Fur with Fe(II), Fe(III), Co(II), and Zn(II) are described. Reconstituted protein was assayed for DNA affinity by gel shift assays. Fur is activated for DNA binding when reconstituted with Fe(III), as well as Fe(II), Zn(II), Co(II), and Mn(II), with little difference in DNA affinity for the different metallo forms of Fur. The affinity of Fur for the different metals was determined and ranges over several orders of magnitude in the following order: Zn(II) ≫ Co(II) > Fe(II) > Mn(II). Only Fe(II) binds with sufficient affinity to activate Fur significantly at physiological metal concentrations, when compared to previously determined total metal concentrations in *Escherichia coli*.

Since the discovery of the ferric uptake regulator (Fur)[1] (*1*), a number of studies have shown that this regulatory protein can be activated for DNA binding by various divalent metal cations (*2*, *3*). Using a cell-free transcription−translation system with β-galactosidase as the reporter gene, one study showed activation of Fur by Fe(II), Co(II), or Mn(II) (*2*). In another report, DNase I footprinting experiments indicated that, in addition to the previous metals, Zn(II), Cu(II), and Cd(II) can also activate Fur for DNA binding (*3*).

The ability of various metals to activate Fur for DNA binding in vitro brings into question the metals that are functional in vivo. Since the physiological metal must bind to and activate Fur within a physiologically relevant concentration range, knowing the affinity of Fur for different metals and the concentrations of those metals in vivo could help in identifying the functional metal. Several estimates of the affinity of Fur for metals have been reported, ranging from 10 to 80 μM, depending on the metal and the method used (*2*, *4*, *5*). However, these estimates of metal affinity were complicated by nonspecific binding of multiple ions to the protein. An accurate determination of this critical parameter is needed to determine the physiological metal for Fur. Additionally, the effect of the oxidation state of iron and metal stoichiometry on the DNA binding activity of Fur remains unclear.

In this study, the affinity of Fur for different metals was determined by metal titration experiments using metal chelators to allow precise control of the free metal ion concentration in solution. Methods for the reconstitution of Fur with different metals were developed, allowing determination of metal stoichiometry and examination of the effect of iron oxidation state on Fur activity. Together, these data and reported intracellular metal concentrations in *Escherichia coli* were used to address the question of which metal binds to and activates Fur under physiological conditions.

MATERIALS AND METHODS

General. All reagents were from commercial suppliers and were used without further purification, except when indicated otherwise. To avoid metal contamination, plasticware was used as much as possible. Where glassware was necessary,

† This work was supported in part by NIH Grant CA26731.

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[1] Abbreviations: BisTris, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IDA, iminodiacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; OD_{600}, optical density at 600 nm; EMSA, gel electrophoretic mobility shift assay; 6-FAM, fluorescein; EPR, electron paramagnetic resonance; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Fur, ferric uptake regulator protein; Zn_1Fur, Fur protein with one Zn(II) atom bound; Zn_2Fur, Fur with two Zn(II) atoms bound; MnFur, Fur with Mn(II) and Zn(II) bound; CoFur, Fur with Co(II) and Zn(II) bound; Fe(II)Fur, Fur with Fe(II) and Zn(II) bound; Fe(III)Fur, Fur with Fe(III) and Zn(II) bound; DtxR, diphtheria toxin regulator.

10.1021/bi0507579 CCC: $30.25
Published on Web 09/22/2005

it was rinsed with concentrated HNO_3 prior to use. Anaerobic procedures were performed in an anaerobic chamber (Coy Laboratory Products, Inc.) with an atmosphere containing ~5% H_2 in Ar.

Protein Expression and Purification. DNA encoding the *E. coli* fur gene in the pMON2064 plasmid was obtained from M. Coy (University of California, Berkeley) (*6*). This plasmid was digested with NcoI and HindIII to excise the gene encoding Fur. Expression of the Fur protein was put under control of the T7 promoter by inserting the fur gene into pET21d (Novagen), which had been digested with the same enzymes. The correct gene sequence and insertion sites were confirmed by DNA sequencing (DNA sequencing facility, University of California, Berkeley). The pET21d/Fur plasmid was transformed into BLR(DE3) cells (Novagen) for protein expression.

Three 1 L cultures of the transformed BLR(DE3) cells were grown in LB medium with 200 μg/mL ampicillin to an OD_{600} of ~0.6. Expression of Fur was induced by addition of IPTG (1 mM) to these cultures. The cultures were shaken for at least 4 h after addition of IPTG before the cells were harvested by centrifugation.

Protein purification was performed using a method similar to that described by Wee et al. (*6*), with several modifications. Approximately 8 g of wet cells was suspended in 25 mL of 50 mM Tris buffer (pH 8.0). To this were added 3 mL of 80% glycerol, 6 mg of Pefabloc (Pentapharm), and 0.1 mg of pepstatin A. This mixture was incubated on ice for 30 min prior to cell lysis by sonication. Cell debris was cleared by centrifugation (15000*g* for 30 min). Streptomycin sulfate (1%, w/v) was added to the supernatant and the mixture gently inverted at 4 °C for 1 h before centrifugation (15000*g* for 30 min) to remove DNA. To the supernatant were added 800 μL of $MgCl_2$ (1 M) and 250 μL of DNase I (5 mg/mL). This mixture was incubated on ice for 30 min and cleared by centrifugation (100000*g* for 45 min).

The supernatant was diluted to 50 mL with 50 mM Tris (pH 8.0) and loaded onto an IDA−agarose (Pierce) column (1.6 cm × 14 cm, ~20 mL of resin), which had been loaded with $ZnCl_2$ and equilibrated with buffer consisting of 20 mM Tris and 500 mM NaCl (pH 8.0) (buffer A). The column was washed sequentially with 50 mL of buffer A, 150 mL of 100 mM BisTris (pH 6.5), and 150 mL of buffer A. Nonspecifically bound protein was eluted with 100 mL of 25 mM imidazole in buffer A. Fur was eluted with 250 mM imidazole in buffer A. Fractions containing Fur were identified by SDS−PAGE. Fur was desalted into buffer consisting of 20 mM HEPES and 100 mM NaCl (pH 7.5) by gel filtration using a column of G-25 resin (2.5 cm × 25 cm) equilibrated with the same buffer. Fractions containing Fur were collected and concentrated to <10 mL using a Vivaspin centrifugal concentrator (10K molecular weight cutoff, Vivascience).

Protein Characterization. Protein concentrations were determined by the Bradford assay (Bio-Rad) using BSA as the standard. Quantitative amino acid analysis (Protein Chemistry Laboratory, Texas A&M University, College Station, TX) indicated that the Bradford assay overestimates the Fur concentration by a factor of 1.5. This correction factor was used for all protein concentration determinations. The metal content of the protein was determined by ICP-AES using a Perkin-Elmer 3000DV spectrometer and commercially available standards. Antibodies to Fur were a gift from T. V. O'Halloran (Northwestern University, Evanston, IL).

Zinc Removal. Zn_1Fur was prepared following the procedure of Althaus et al. (*7*). Purified Fur was dialyzed overnight against buffer consisting of 100 mM EDTA, 20 mM HEPES, 100 mM NaCl, and 5% glycerol (pH 7.5). EDTA was removed by dialysis against the same buffer without EDTA followed by three sequential concentration and dilution cycles in an Ultrafree-0.5 centrifugal filter device (10K molecular weight cutoff, Millipore).

Metal Reconstitution. For all metals except Co(II), incorporation of the metal was accomplished by incubating Zn_1-Fur (50 μM, 200 μL) with the appropriate metal salt (200−250 μM) for 20 min on ice. Excess metal was removed by desalting with a PD-10 column (Amersham). In the case of Fe(II), the protein sample was made anaerobic prior to metal addition. The reconstitution was carried out in an anaerobic chamber, and ascorbic acid (200 μM) was included in the solution. In the case of Fe(III), $FeCl_3$ was dissolved in water containing a 10-fold excess of citrate adjusted to pH 7.0. The protein concentration was reduced to 25 μM. The reconstitution and desalting steps were carried out with limited light to avoid possible photoreduction of Fe(III). For Zn(II) incorporation, 2.3 equiv of $ZnCl_2$ (115 μM) was used. Incorporation of Co(II) was accomplished by dialyzing Zn_1-Fur (2.5 mL at 80 μM) against 250 mL of HEPES buffer containing 50 μM $CoCl_2$ overnight at 4 °C. Excess metal was removed by continued dialysis against HEPES buffer without $CoCl_2$, followed by concentration and dilution of the protein solution three times in an Ultrafree-0.5 centrifugal filter device (10K molecular weight cutoff, Millipore).

Oxidation of Fe(II)Fur. Fe(II)Fur (50 μM) was oxidized by adding $K_3Fe(CN)_6$ (1 mM) to the solution. After 20 min, the $K_3Fe(CN)_6$ was removed by desalting with a PD-10 column (Amersham) followed by two rounds of concentration and dilution. This procedure was carried out with limited light to avoid photoreduction of Fe(III).

Determination of the Iron Oxidation State in Fur. The amount of ferrous iron in Fur was determined by the ferrozine assay (*8*). Ferrozine dye was added anaerobically to a Fur solution without addition of reductant or denaturant. An immediate color change was observed. The absorbance due to the Fe(II)−ferrozine complex was measured at 562 nm (ϵ = 27 900 M^{-1} cm^{-1}) (*9*). When the solution was kept anaerobic, the amount of Fe(II) in Fe(II)Fur as determined by the ferrozine assay was consistent with the total amount of iron as determined by ICP-AES analysis.

The amount of ferric iron in Fur was determined by subtracting the amount of ferrous iron, as determined by the ferrozine assay, from the amount of total iron, as determined by ICP-AES. The presence of ferric iron bound to Fur was confirmed by EPR using a Varian E-109 system equipped with a standard TE102 cavity and a Heli-tran liquid helium cryostat (Air Products). Samples were placed in quartz EPR tubes and frozen in isopentane cooled with liquid N_2. Spectra were obtained at 8 K with a microwave frequency of 9.25 GHz, a microwave power of 2 mW, a modulation amplitude of 20 G, a modulation frequency of 100 kHz, a scan range of 4000 G, and a scan time of 4 min. The intensity of the EPR signal was compared to the intensity of a sample of

$FeCl_3$ (40 μM, the same concentration as the protein) dissolved in sodium citrate (400 μM).

Gel Electrophoretic Mobility Shift Assays. Gel mobility shift assay procedures were modified from published procedures (*10*, *11*). The DNA probe used for this study was made in the following manner. Approximately equal amounts of complementary oligonucleotides (forward, 5′-GAAGGAGATATACCATGGATAATGATAATCATTATCTCGAGCACC-6-FAM-3′; reverse, 5′-GGTGGTGCTCGAGATAATGATTATCATTATCCATGGTATATCTCC-3′, Integrated DNA Technologies) containing the 19 bp Fur box (underlined) were mixed and annealed by heating to 95 °C and cooling slowly. Single-stranded DNA was separated from double-stranded DNA by HPLC using a Zorbax Bio Series GF-250 column (Agilent) using a mobile phase consisting of 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM $MgCl_2$. The double-stranded oligonucleotide was ^{32}P-labeled using T4 polynucleotide kinase (Invitrogen), following the manufacturer's instructions.

Radiolabeled DNA and Fur were mixed in a buffer consisting of 20 mM BisTris/borate (pH 7.0), 1 mM $MgCl_2$, 40 mM KCl, and 5% (v/v) glycerol (binding buffer). To determine the DNA binding activity of different metallo forms of Fur, metal-reconstituted Fur was used in the reactions and the binding buffer was supplemented with the chloride salt (100 μM) of the metal in the protein (200 μM in the case of Mn). After 20−30 min at room temperature, the reaction mixtures were loaded onto a 5% (29:1) nondenaturing acrylamide gel.

The gel (14 cm × 14 cm × 0.15 cm) was prepared with 20 mM BisTris/borate (pH 7.0) and 500 μM EDTA. The EDTA was removed from the gel by prerunning the gel for 6 h at 200 V with buffer containing 20 mM BisTris/borate (pH 7.0) and no EDTA. Protein was electrophoresed at 225 V for 80 min. After electrophoresis, the gel was transferred to filter paper and dried before being imaged by phosphorescence using a Typhoon 9410 phosphoimager (Amersham Biosciences). The K_D was estimated by visual inspection from the imaged gel.

In the case of Fe(II), the binding reactions were carried out in an anaerobic chamber. The binding buffer was supplemented with 50 μM sodium dithionite to ensure complete reduction of the sample. The ferrozine assay was used to confirm the presence of a stoichiometric amount of Fe(II) before the binding reactions were initiated. After the gel polymerized, it was transferred into an anaerobic chamber. The gel was prerun and electrophoresed in the anaerobic chamber using anaerobic buffers.

Metal Titration Assays. Metal titrations were performed following the method of Outten and O'Halloran (*12*). Free metal concentrations were calculated using HySS (*13*) with association constants found in the IUPAC stability constants database, version 4.05 (Academic Software). Absorbance spectra were obtained on a Cary 300 Bio spectrophotometer maintained at 25 °C. Fluorescence spectra were obtained on a Fluoro-Max2 (Jobin Yvon Horiba) fluorimeter thermostated to 25 °C. Zn_1Fur (5 μM) in HEPES buffer was placed in a fluorescence cuvette fitted with a ground glass joint to allow attachment of a threaded plunger syringe (Hamilton). For the titration with Fe(II), the cuvette and syringe were assembled in the anaerobic chamber to exclude oxygen. Initial absorbance and emission spectra were obtained. The syringe was filled with a 1 mM solution of the appropriate metal salt [3 mM in the case of Mn(II)] and inserted into the cuvette. Metal was titrated into the protein solution in 1.25 μL aliquots. Absorbance and emission spectra were obtained 3 and 7 min after each metal addition, respectively. Metal incorporation was followed by the quenching of tyrosine fluorescence (excitation at 276 nm and emission at 305 nm). Data were fit to a four-parameter sigmoidal dose−response curve (eq 1) with no parameters constrained

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + 10^{(x - \log K_D)B}} \quad (1)$$

where x is the log of the molar metal concentration, y is the fluorescence intensity at 305 nm, K_D is the binding constant for the binding of metal to Zn_1Fur, and B is the Hill coefficient. After an initial fit of the data, the K_D for Fur from the fit was introduced back into HySS to recalculate the concentration of the free metal. The new concentrations were used to replot the titration curve and recalculate K_D. This process was repeated until the K_D did not change significantly. For each metal, the total change in K_D was a factor of less than 2 [in the case of Zn(II)] and usually closer to 10% of the final value.

RESULTS

Protein Purification

Fur was purified as previously reported (*6*) with several modifications. First, the ammonium sulfate precipitation steps were eliminated. The streptomycin sulfate precipitation was found to be important as elimination of this step left a number of contaminating proteins in the mixture that were not removed by chromatography (data not shown). From this procedure, 30 mg of protein was obtained per liter of cell culture and determined to be >95% pure by SDS−PAGE. Protein purified in this manner was found to contain 2−3 equiv of Zn and no Fe. Quantitative amino acid analysis indicated that the Bradford assay, with BSA as a standard, overestimates the protein concentration by a factor of 1.5. This factor was used to correct all protein concentration determinations.

Preparation of Zn_1Fur

Dialysis against EDTA provided Fur containing between 0.9 and 1.2 equiv of Zn, Zn_1Fur, as reported by Althaus et al. (*7*). This metal is thought to serve a structural role in Fur. It is tightly bound and is only removed by denaturation of the protein. Complete removal of EDTA was required to allow reconstitution of Fur with other metals.

Metal Reconstitution of Zn_1Fur

Fe(II) Reconstitution. A general procedure for reconstitution of Fur with metal is to add a 5-fold excess of the metal to Zn_1Fur (50 μM). Unbound metal is removed by gel filtration after 15−20 min to isolate the metal−protein complex. Using $Fe(NH_4)_2(SO_4)_2$ as the metal, this procedure resulted in a protein containing 1.3 equiv of Fe and 1.2 equiv of Zn, Fe(II)Fur (Table 1). The ferrozine assay indicated that essentially all of the Fe was Fe(II).

Table 1: Metal Stoichiometry after Reconstitution

	no. of metals/monomer[a]	
metal	M	Zn(II)
Zn(II)		2.3 ± 0.1
Co(II)	1.3 ± 0.1	0.83 ± 0.1
Fe(II)	1.3 ± 0.3	1.2 ± 0.3
Fe(III)	1.71 ± 0.1	1.01 ± 0.02
Mn(II)	0.21 ± 0.01	0.67 ± 0.03

[a] The error is the propagated error from the standard deviations of triplicate determinations of the protein and metal concentrations. The protein concentrations were approximately 5 μM.

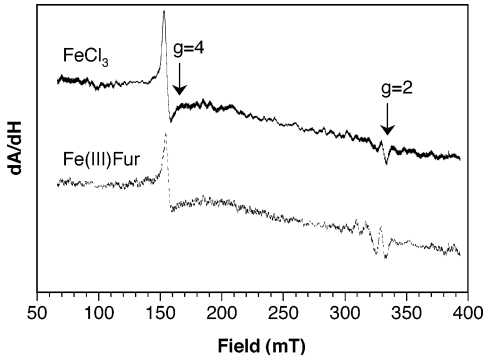

FIGURE 1: X-Band EPR spectra of Fe(III)Fur and $FeCl_3$. The bottom spectrum is of Fe(III)Fur (42 μM), and the top spectrum is of $FeCl_3$ (40 μM) with 200 μM sodium citrate. Both spectra were recorded under the same conditions to allow comparison of the signal intensities. The spectra are offset for clarity. Approximate *g* values are indicated. Spectrometer parameters were as follows: 9.25 GHz microwave frequency, 2 mW power, 8 K, and 20 G modulation amplitude.

Mn(II) Reconstitution. Using $MnCl_2$, this procedure resulted in a protein containing 0.2 equiv of Mn and 0.7 equiv of Zn (Table 1). The inability to isolate MnFur is likely due to the low affinity of Fur for Mn(II), as shown below. It should be noted that although MnFur could not be isolated, Mn(II) does bind to and activate Fur for DNA binding (also shown below).

Zn(II) Reconstitution. Zn_2Fur can be prepared by limiting the added Zn to a 2-fold excess over protein rather than a 5-fold excess. A larger excess of zinc, in the absence of DTT, causes Fur to precipitate. After gel filtration, the protein was found to have 2.3 equiv of Zn(II) (Table 1). These results are consistent with a previous procedure for Zn(II) incorporation using a 5−10-fold excess of Zn(II) and a 20-fold excess of DTT (*7*). The observation of excess metal bound to Fur (>2 equiv) is a result similar to the metal content of the protein as purified. The additional metal-binding site has not been characterized, but a recent crystal structure of the Fur homologue from *Pseudomonas aeruginosa* shows three Zn atoms per Fur monomer. The extra Zn atom is located in the dimer interface and does not seem to be involved in protein function (*14*).

Co(II) Reconstitution. Attempts to incorporate Co(II) into Zn_1Fur by incubation with 200 μM $CoCl_2$ resulted in the precipitation of most of the protein. Decreasing the metal concentration eliminated precipitation but required a greater volume of metal solution to provide sufficient metal to Fur. Dialysis of Zn_1Fur against buffer containing 50 μM $CoCl_2$ resulted in Fur with 1.3 equiv of Co and 0.8 equiv of Zn, CoFur (Table 1).

Fe(III) Reconstitution. Fe(III) was incorporated into Fur using two different methods. In the first method, Fe(III) was incorporated directly into Zn_1Fur by adding the protein (25 μM final concentration) to a solution containing 250 μM $FeCl_3$, which had been dissolved in a 10-fold excess of sodium citrate (pH 7.0). Unbound metal was removed by gel filtration. Prepared this way, the protein was found to have 1.7 equiv of Fe and 1.0 equiv of Zn, Fe(III)Fur. Addition of ferrozine to this protein did not produce an absorbance at 562 nm, indicating no Fe(II) was present. The presence of Fe(III) was confirmed by EPR spectroscopy. Figure 1 shows the EPR spectrum of Fe(III)Fur (42 μM) and a spectrum of $FeCl_3$ (40 μM) dissolved in sodium citrate (400 μM), for comparison. Both spectra show major signals near $g = 4$ and smaller signals at $g = 2$. The magnitudes of the signals in both spectra are similar, indicating a similar amount of Fe(III) in each sample. A second method for preparing Fur containing Fe(III) is oxidation of Fe(II)Fur with $K_3Fe(CN)_6$. Fe(III)Fur, prepared by this method, was unreactive with ferrozine and had an EPR spectrum similar to that of the protein prepared by direct incorporation of Fe(III). Regardless of the method of preparation, Fe(III)Fur is activated for DNA binding (see below).

Gel Electrophoretic Mobility Shift Assays

The ability of each metallo form of Fur to bind DNA was determined by gel mobility shift assays. Gel shift assays with Fur have been used in previous studies, but DNA dissociation constants have been reported for only Zn_2Fur (*7*). A K_D for MnFur can be estimated from ref *11*.

Initially, the aerobactin (*iucA*) promoter region was used for these studies, as described previously (*11*). However, multiple shifts in DNA mobility have been reported with this promoter (*11*) and were observed in this study (data not shown). The nature and number of Fur binding sites in this promoter region are the subject of some debate (*3*, *11*, *15*−*17*). The multiple shifts are likely due to multiple Fur binding sites within this promoter. To avoid these problems, a synthetic 45 bp oligonucleotide was used in this study.

Although no transition metals were detected in any of the components used for the mobility shift assays by ICP-AES, enough metal was present to activate Zn_1Fur for DNA binding. By including EDTA in the gel and prerunning the gel for 6 h, we removed the metal, and Zn_1Fur (300 nM) did not produce a mobility shift (data not shown). Using the gel prepared this way, it was necessary to include the metal salt (100 μM) in the binding reaction to observe a mobility shift with reconstituted protein (200 μM in the case of Mn^{2+}). This is likely due to the dissociation of the metal from the protein at the low protein concentrations used for the mobility shift assays (see below for the dissociation constants for dissociation of the metal from the protein).

Dithionite (50 μM) was added to Fe(II)Fur prior to diluting the protein for the mobility shift assays to ensure the presence of Fe(II) in Fur. The ferrozine assay confirmed the presence of a stoichiometric amount of Fe(II).

The results of the mobility shift assays for Fur with the various metal ions bound are shown in Figure 2, and summarized in Table 2. All of the metals that were studied were able to activate Fur for DNA binding with similar dissociation constants, ranging between 20 and 60 nM.

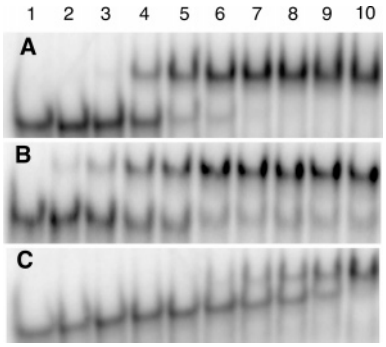

FIGURE 2: Electrophoretic mobility shift assay of Fur with various metals: (A) CoFur, (B) Fe(II)Fur, and (C) Fe(III)Fur. DNA concentrations were ~0.7 nM. Protein concentrations were as follows: lane 1, 0 nM; lane 2, 5 nM; lane 3, 10 nM; lane 4, 20 nM; lane 5, 30 nM; lane 6, 40 nM; lane 7, 50 nM; lane 8, 60 nM; lane 9, 70 nM; and lane 10, 80 mM.

Table 2: Binding Constants for Binding of Different Metallo Forms of Fur to DNA

	K_D (nM)[a]	K_D (nM)[b]
CoFur	20	
Zn_2Fur	40	20[c]
MnFur	20	<20[d]
Fe(II)Fur	20	
Fe(III)Fur	60	

[a] Binding constants determined by visual inspection of data presented in Figure 2. [b] Previous estimates of the Fur−DNA dissociation constant. [c] Taken from ref *7*. [d] Taken from ref *11*.

Importantly, this study shows that both Fe(II) and Fe(III) are capable of activating Fur for DNA binding.

Titration of Metals into Fur

The dissociation constants for dissociation of different metal cations from Fur may identify which cations are functional in vivo. Dissociation constants for the different metals were measured by monitoring the quenching of tyrosine fluorescence as the metal was titrated into a solution of Fur (5 μM). Because of the relatively high affinity of Fur for metal and to accurately control the concentration of free metal in the titrations, a competition between the protein and a chelator was used (*18*, *19*). For most of the metals that were studied, it was found that using 1 mM citrate as the chelator provided the appropriate concentration of free metal. However, because the K_D for Zn(II) was lower than the values for the other metals, 5 mM IDA was used as the chelator. HySS (*13*) was used to calculate the concentration of free metal using constants from the IUPAC stability constants database, version 4.05 (Academic Software). None of the four parameters of a sigmoidal dose−response curve was constrained in fitting the data. Since the change in the fluorescence of the protein is likely due to a conformational change as metal binds, the midpoint of the curve was taken to be the K_D.

The titration curves obtained for Co(II), Zn(II), Mn(II), and Fe(II) are shown in Figure 3, and the dissociation constants determined from the fitted lines are shown in Table 3. The K_D values were found to range from 0.14 nM to 24 μM, in the following order: Zn(II) ≫ Co(II) > Fe(II) > Mn(II). The UV−vis spectrum of each titration was monitored during metal titrations to detect protein precipitation.

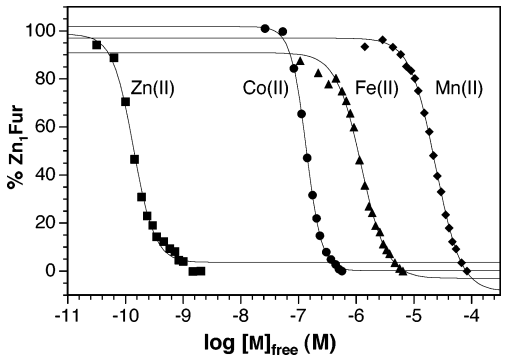

FIGURE 3: Fluorescence quenching titration curve for addition of M(II) to Fur. A solution of the metal salt was titrated into a solution containing 5 μM Zn_1Fur and metal chelator in HEPES buffer. The fluorescence emission intensity (excitation at 276 nm and emission at 305 nm) was measured 7 min after each addition of metal solution. The data were fit to eq 1. The concentrations of the metal salts were as follows: 1 mM $CoCl_2$ with 1 mM sodium citrate (●), 1 mM $ZnCl_2$ with 5 mM IDA (■), 3 mM $MnCl_2$ with 1 mM sodium citrate (◆), and 1 mM $Fe(NH_4)_2(SO_4)_2$ with 1 mM sodium citrate (▲).

Table 3: Affinity of Fur for Different Metals

	K_D (M)[a]	Hill coefficient	$[M]_{total}$ (M)[b]	K_D (M)[c]
Co(II)	1.5×10^{-7}	3.3	$<10^{-7}$	3.6×10^{-5} [d]
Zn(II)	1.4×10^{-10}	2.4	10^{-4}	
Mn(II)	2.4×10^{-5}	2.1	10^{-5}	8.5×10^{-5} [d] 8.0×10^{-5} [e]
Fe(II)	1.2×10^{-6}	2.0	10^{-4}	5.5×10^{-5} [d] 1×10^{-5} [f]

[a] Errors are ≤5%. [b] Taken from ref *12*. [c] Previous estimates of metal binding constants. [d] Taken from ref *4*: six Co atoms/Fur monomer and two Fe atoms/Fur monomer. [e] Taken from ref *5*. [f] Taken from ref *2*.

For the divalent metals that were studied, no significant baseline shift was observed, nor was there a significant change in the UV−vis spectrum. The titration could not be performed with Fe(III) due to the absorbance of the Fe(III)−citrate complex (data not shown), which caused a significant loss of fluorescence intensity and prevented completion of the titration with this ion. Included in Table 3 are the Hill coefficients from the curve fits. In each case, the Hill coefficient is greater than one, suggesting cooperative binding of multiple metal ions. Since Fur is a dimer, it is likely that a conformational change in one monomer, due to metal binding, would affect the other monomer, leading to cooperative metal binding.

DISCUSSION

The ability of Fur to be activated for DNA binding by different divalent cations has been shown previously. It has also been shown that $FeSO_4$ activates Fur for DNA binding (*2*, *3*, *20*); however, the tendency for Fe(II) to oxidize brings into question the oxidation state required for Fur activation. This report shows that Fur is activated by both Fe(II) and Fe(III). The ability of the same metal-binding site to bind both a di- and trivalent cation is unexpected. It is possible that Fe(III) binds to a different site than the divalent cations. However, this appears to be unlikely since oxidation of Fe(II)Fur results in Fe(III)Fur which is active for DNA binding. Additionally, attempts to incorporate Co(II) into Fe(III)Fur resulted in exchange of Co(II) for Fe(III), suggesting that they have at least one site in common. Furthermore, it

is not obvious how the extra metal binding sites from the *P. aeruginosa* Fur crystal structure could be involved in protein activation or that they would bind Fe(III) rather than a divalent cation (*14*).

Since Fur can be reconstituted with several different metal cations and these metals are equally effective in activating Fur for DNA binding, the identity of the metal responsible for Fur activation in vivo is brought into question. Although thermodynamics alone may not control insertion of metal into Fur, the affinity of Fur for different metals, when compared to the concentration of those metals in vivo, may be suggestive of the functional metal. If the role of Fur in vivo is to sense the concentration of iron in the cell, a specific metallochaperone would not be needed. It is possible, however, that Fur competes with other proteins for loosely bound metal. In this case, the K_D of metal for Fur, relative to the K_Ds of metal for other proteins, will dictate which protein is metal-bound. A previous estimate of iron binding to Fur (10 μM) was based on the amount of cold acid-soluble iron in a cell-free transcription/translation mix (25 μM iron) and the assumption that approximately half of that iron would be available for binding to Fur (*2*). Equilibrium dialysis was used to determine binding constants for Fe(II) (30 μM), Mn(II) (85 μM), and Co(II) (36 μM) (*4*). However, since multiple ions bound Fur in those studies [e.g., two Fe(II)/Fur and six Co(II)/Fur], the meaning of the binding constants is unclear. Another estimate of the K_D for binding of Mn to Fur ($\sim$80 μM) was made by Mn titration, similar to the method in this report (*5*). However, the reported titration was a direct titration rather than a competition experiment. A competition experiment allows for more accurate control of the concentration of free metal, especially at low metal concentrations (*12*), and therefore a more accurate determination of the K_D.

Of the metals examined in this study, Zn(II) has the highest affinity for Fur (1.4×10^{-10} M) and could activate Fur in vivo; however, the concentration of free zinc in *E. coli* ($\sim 10^{-15}$ M) (*12*) is much lower than the K_D of Zn for Fur. Although estimates of the intracellular concentrations of free Co(II) and Mn(II) are not available, the total concentrations of these metals in *E. coli* have been determined (see Table 3 and ref *12*). The constants for binding of these metals to Fur could be physiologically relevant. In fact, previous studies have shown that addition of $MnCl_2$ to the growth medium results in Fur activation in vivo (*21*). However, since the *total* concentrations of Co and Mn are at the K_D values for these metals, it is unlikely that either of these metals is free in solution at concentrations sufficient to activate Fur in vivo.

As with Co(II) and Mn(II), the intracellular concentration of free iron in *E. coli* is not known. However, the total concentration of iron in *E. coli*, whether grown on minimal or rich medium, is 10^{-4} M (*12*). This value represents the total amount of iron in *E. coli* in all forms, bound and free, and overestimates the amount of iron available to bind to Fur. The concentration of "loosely bound" iron has been estimated to be $\sim$10 μM (*22*); thus, a K_D of 1.2 μM for binding of Fe(II) to Fur is within a physiologically relevant concentration range. Since the estimates of total and loosely bound iron are sufficient for activation of Fur and repress iron uptake, it seems likely that iron is the metal that activates Fur under physiological conditions.

From a physiological perspective, Fur may act in a protective fashion to prevent iron toxicity. That is, when iron levels increase above a threshold, Fur is activated to attenuate iron import and prevent iron overload. In this role, distinguishing between Fe(II) and Fe(III) would not be necessary, as excessive concentrations of either ion can be toxic. In a functionally similar protein, the diphtheria toxin repressor (DtxR), Fe(II), but not Fe(III), activates the protein for DNA binding (*23*). In contrast with Fur, the role of DtxR may be to sense iron starvation and to increase iron import via siderophore synthesis.

The ability of Fur to be activated by metals other than iron may have physiological implications. One possibility is that Fur is used to sense more than one metal in bacteria and the different metallo forms of Fur control different genes by binding preferentially to different DNA sequences. An examination of the affinity of different metallo forms of Fur for different DNA sequences has not been reported. Alternatively, elevated levels of other metals could interfere with normal iron regulation by activating Fur inappropriately, thus shutting down iron import and leading to iron starvation. This phenomenon is illustrated by the observation that Mn(II) is toxic to *fur*$^+$, but not *fur*$^-$, *E. coli* strains (*21*).

CONCLUSIONS

This report describes methods for reconstitution of Fur with various metal cations. The ability of different metallo forms of Fur to bind DNA was evaluated. Little difference in DNA affinity was found for the different metal ions that were studied. Notably, it was found that both Fe(II) and Fe(III) activate Fur for DNA binding. Metal to protein dissociation constants were also determined for each of the metals that were studied, and found to range over several orders of magnitude. The dissociation constant for Fe(II) was found to be within a physiologically relevant range, suggesting that iron is the physiological metal for Fur.

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BI0507579