Functional characterization of the dimerization domain of the ferric uptake regulator (Fur) of *Pseudomonas aeruginosa*

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The functional properties of the recombinant C-terminal dimerization domain of the *Pseudomonas aeruginosa* Fur (ferric uptake regulator) protein expressed in and purified from *Escherichia coli* have been evaluated. Sedimentation velocity measurements demonstrate that this domain is dimeric, and the UV CD spectrum is consistent with a secondary structure similar to that observed for the corresponding region of the crystallographically characterized wild-type protein. The thermal stability of the domain as determined by CD spectroscopy decreases significantly as pH is increased and increases significantly as metal ions are added. Potentiometric titrations (pH 6.5) establish that the domain possesses a high-affinity and a low-affinity binding site for metal ions. The high-affinity (sensory) binding site demonstrates association constants (K_A) of $10(\pm 7) \times 10^6$, $5.7(\pm 3) \times 10^6$, $2.0(\pm 2) \times 10^6$ and $2.0(\pm 3) \times 10^4 \text{ M}^{-1}$ for Ni^{2+} , Zn^{2+} , Co^{2+} and Mn^{2+} respec-

tively, while the low-affinity (structural) site exhibits association constants of $1.3(\pm 2) \times 10^6$, $3.2(\pm 2) \times 10^4$, $1.76(\pm 1) \times 10^5$ and $1.5(\pm 2) \times 10^3 \text{ M}^{-1}$ respectively for the same metal ions (pH 6.5, 300 mM NaCl, 25 °C). The stability of metal ion binding to the sensory site follows the Irving–Williams order, while metal ion binding to the partial sensory site present in the domain does not. Fluorescence experiments indicate that the quenching resulting from binding of Co^{2+} is reversed by subsequent titration with Zn^{2+} . We conclude that the domain is a reasonable model for many properties of the full-length protein and is amenable to some analyses that the limited solubility of the full-length protein prevents.

Key words: analytical ultracentrifugation, CD spectroscopy, ferric uptake regulator (Fur), potentiometric titration, *Pseudomonas aeruginosa*, thermal stability.

INTRODUCTION

Bacteria must often contend with nutrient-poor environments where their survival hinges on tight control over efficient nutrient uptake pathways. Iron, for example, is a nutrient that can limit growth severely when it is scarce. Many bacteria cope with iron-poor conditions by secreting low-molecular-mass chelators or siderophores that scavenge this essential mineral from the local environment for more efficient absorption into the cell via outer membrane receptors [1–3]. Control of many of these pathways is accomplished genetically through the inactivation of Fur (ferric uptake regulator) that is believed to act as an Fe^{2+} -binding transcription repressor [3–5]. In *Escherichia coli*, members of the Fur family regulate the expression of more than 100 genes that function in processes as varied as the biosynthesis and transport of siderophores (> 60 genes [3,6]), the expression of virulence factors (13 genes [3]), the alleviation of oxidative and NO-induced stress [7], and the inhibition of ferritin production through the expression of RyhB [8]. Similarly, in the opportunistic pathogen *Pseudomonas aeruginosa*, Fur regulates the expression of at least 27 genes that are necessary to counteract oxidative stress or to trigger the transcription of virulence genes through the *pvdS* σ factor [2,6,9,10]. Finally, Fur is known to be responsible for similar functions in other organisms including *Salmonella* Typhimurium [11], *Helicobacter pylori* [12,13] and some mycobacterial strains [14].

Considering the importance and widespread occurrence of Fur proteins, surprisingly little is known about the mechanism(s) of Fur activity. Early models envisioned Fur acting as a classical repressor that binds to its target DNA, a 19-bp sequence com-

monly known as the ‘iron-box’ or ‘Fur-box’, whenever a sensory or regulatory Fe^{2+} -binding site per monomer is occupied [4,15–17]. However, the discovery of a structural Zn^{2+} -binding site in *E. coli* Fur that is distinct from the sensory Fe^{2+} -binding site [18–20] calls into question the role of Fe^{2+} as a co-repressor. Binding of Zn^{2+} at this structural site is required for the formation of a Fur–DNA complex, but the same is not necessarily true for Fe^{2+} at the sensory site because Fur can bind DNA with high affinity even in the absence of iron provided that Zn^{2+} is present [20]. This observation could explain earlier results that indicated that metal ions other than Fe^{2+} (e.g. Mn^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+}) can promote binding of Fur from a wide variety of organisms to their respective DNA recognition sequences [15,21,22].

Much of the uncertainty regarding the mechanism of Fur activity concerns the fundamental issue of metal ion binding and the linkage of metal ion binding to functional properties of the protein. The recent elucidation of the three-dimensional structure of *Ps. aeruginosa* Fur by X-ray crystallography [23] represents a major advance towards solving this problem. This structure establishes that *Ps. aeruginosa* Fur is prone to dimerization and that each monomer can bind two Zn^{2+} ions, as is the case for *E. coli* Fur [20]. On the basis of this structure and spectroscopic experiments, the proposed ‘sensory’ metal-ion-binding site is located completely within the dimerization domain of each monomer and exhibits a distorted octahedral geometry in which His^{86} , Asp^{88} , Glu^{107} and His^{124} co-ordinate such that the carboxy group of Asp^{88} serves as a bidentate ligand and a water molecule completes the co-ordination environment (site 1 [23]). The other Zn^{2+} ion binds at the proposed ‘structural’ metal-ion-binding site located at the interface of the dimerization and DNA-binding domains.

Abbreviations used: c(M), molar mass distribution; c(s), sedimentation coefficient distribution; Fur, ferric uptake regulator; MBP, maltose-binding protein.

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	62	▼▼	80	90	••	100
<i>Ec</i>	DDAGIVTRH	NFEGGKSVFE	LTQHHHDHL	ICLDGKQVIE		
<i>Pa</i>	<u>G</u> SEAAGLVVRH	NFDGGHAVFE	LADSGHHDM	VCVDTGEVIE	*	
	1	10	20	30		
	110	120	130	140	148	
<i>Ec</i>	FSDDSIIEARQ	REIAAKHGIR	LTNHSLYLYG	HCAEGDCRED	EHAHEGKN	
<i>Pa</i>	FMDAEIEKRQ	KEIVRERGFE	LVDHNLVLYV	RKKK		
	40	50	60	73		

Figure 1 Comparison of amino acid sequences of Fur from *E. coli* (*Ec*) and *Ps. aeruginosa* (*Pa*)

The numbers above the *E. coli* Fur sequence denote the numbering scheme with respect to full-length Fur, and the numbers below the *Pseudomonas* sequence represent the sequence of the C-terminal domain. The residues (Gly⁷⁵ and Ser⁷⁷) that have been identified as the domain boundary in *E. coli* Fur [24,25] are identified (▼), as are the cysteine residues (●) of the same protein that co-ordinate a Zn²⁺ ion. The cysteine residue that was mutated to serine in the FurC* construct is indicated by the asterisk above the *Pseudomonas* sequence. The Gly-Ser dipeptide underlined at positions –1 and –2 at of the *Ps. aeruginosa* sequence is the remnant of the thrombin cleavage site (see the Experimental section).

The co-ordination environment observed at this site exhibits a tetrahedral geometry in which the Zn²⁺ is bound to the side chains of His³², Glu⁸⁰, His⁸⁹ and Glu¹⁰⁰ (site 2 [23]).

Although supporting XANES (X-ray near-edge spectrum) data are in agreement with the Zn²⁺ content of the X-ray structure [23], the structure conflicts with the results from previous investigations. In one analytical study, for example, metal-free Fur from *Ps. aeruginosa* was found to bind just one Zn²⁺ ion [24]. In studies of *E. coli* Fur, at least two cysteine residues were found to be involved in the co-ordination of Zn²⁺ at one of the two metal-ion-binding sites [18–20]. In contrast, the sole cysteine residue in the *Ps. aeruginosa* sequence does not appear to participate in metal ion binding [23]. Thus it could be argued that one of the two Zn²⁺ binding sites observed in the crystal structure may result from the crystallization conditions used (i.e. [Zn²⁺] = 10 mM).

On the basis of proteolysis [25] and gene fusion [26] experiments conducted with *E. coli* Fur and on the basis of sequence alignment of the two species of Fur (Figure 1), we have designed, expressed and characterized a domain of the *Ps. aeruginosa* Fur that is comprised of the 73 C-terminal residues and thus constitutes the dimerization domain of the protein. This domain also includes all of the residues responsible for binding of metal ions at the proposed sensory metal-ion-binding site and all but one of the residues involved in the structural metal-ion-binding site

(Figure 2). The dimerization domain has been prepared and characterized in the present study to overcome the limited solubility that is characteristic of the wild-type protein and, thus, to facilitate characterization of the metal ion binding properties of the sensory binding site. The use of structural domains in this manner has led to significant insights regarding the functional properties of other metalloregulatory DNA-binding proteins [27,28]. Recent success in application of this strategy to the study of the N-terminal DNA-binding domain of Fur from *E. coli* demonstrates the feasibility of this approach in the study of the family of Fur proteins as well [29].

EXPERIMENTAL

Protein preparation

The C-terminal domain of the *Ps. aeruginosa* Fur gene (FurC) was amplified from plasmid pMAL-c2 (24) with primers GBB (5'-AGTGGATCCCTGGTGCCACGCGG TTCTGAGGCCGCCGCGCCTGGTGGTG-3') and GBE (5'-GAGGAATTCCTACTTC TTCTTGCGCACGTAG-3'). The BamHI/EcoRI-digested PCR product was subcloned into the expression vector pET30-GBfusion1 immediately behind a thrombin cleavage site and the gene for the B1 domain of Protein G [30] to yield the plasmid pET30-GB1FurC. Furthermore, the wild-type cysteine residue (residue 28 in the FurC sequence) was mutated to serine by the overlap extension PCR method [31], and this mutated gene (FurC*) was reintroduced into pET30-GB1FurC as described above. The fidelity of the resulting fusion gene was verified by sequencing the entire open reading frame. The gene and the 17 kDa fusion protein it encodes are hereafter referred to as GB1-FurC*.

E. coli BL21(DE3)::pET30-GB1-FurC* were grown to a D_{600} of 0.6 at 37°C in liquid LB (Luria–Bertani) medium (5 litres) supplemented with kanamycin (50 µg/ml). Protein expression was induced by addition of IPTG (isopropyl β-D-thiogalactoside) to 0.5 mM, and the cultures were incubated for 3–4 h further. Cells were harvested by centrifugation (15 min, 7000 g), washed in 50 ml of Buffer A (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 10 mM NaCl), and resuspended in 100 ml of Buffer B (Buffer A plus 1 mM dithiothreitol and 1 mM PMSF). Lysozyme, DNase I and RNase I were added (100, 5 and 2 mg respectively per 100 g of cell pellet), and the mixture was incubated on ice for 2–4 h with gentle shaking before two cycles of flash-freezing in liquid nitrogen and thawing at 4°C. The cell lysate was cleared by centrifugation (20 min, 11000 g), and the resulting solution was applied to a DE52 cellulose (Whatman) ion-exchange column (2.5 cm × 20 cm) equilibrated in Buffer C (50 mM Tris/HCl, pH 8.0). The protein was eluted with a linear NaCl gradient

Figure 2 Stereo diagram of the main chain of wild-type Fur as determined by X-ray diffraction analysis [23]

The portion of the protein that comprises the FurC* (dimerization) domain studied in the present study is shown in dark grey, and the portion that is lacking in this domain is shown in light grey. The sensory and structural metal ion-binding sites described by Pohl et al. [23] are indicated.

(0–500 mM) in Buffer C. GB1-FurC*-containing fractions were identified by SDS/PAGE and applied to a 2.5 cm × 14 cm Ni-IMAC [immobilized metal ion (Ni²⁺) affinity chromatography] Sepharose (Amersham Biosciences) column equilibrated in Buffer D (50 mM Tris/HCl, pH 7.4, and 300 mM NaCl). The column was washed with a linear imidazole gradient (0–500 mM) in Buffer D, and fractions containing GB1-FurC* were pooled and dialysed extensively in Buffer C supplemented with NaCl and CaCl₂ (100 and 2.5 mM respectively). Imidazole was recrystallized from toluene to eliminate background absorbance at 280 nm.

The fusion protein was incubated with thrombin (3–5 units of thrombin/1 mg of protein; Haematologic Technologies, Burlington, VT, U.S.A.) at 21 °C for 16–32 h to remove the GB1 sequence to produce FurC* with the additional Gly-Ser dipeptide [molecular mass 8503 a.m.u. (atomic mass units)] from the thrombin cleavage sequence at the N-terminus. When the reaction was complete as determined by SDS/PAGE analysis, the reaction mixture was passed over a fresh Ni-IMAC column that retained the FurC* for elution with 500 mM imidazole in Buffer D. This protein was concentrated (Centriprep-10; Millipore) and exchanged into Buffer E (5 mM Hepes, pH 6.5, and 300 mM NaCl) before separating any uncleaved material by gel-filtration chromatography (Sephadex G50, 2.6 cm × 90 cm).

In preparation and handling of metal-free samples of FurC*, all containers and pipettes were washed with acid and all solutions were treated with a chelating resin [Amberlite IRC-718 (ICN Bio-medicals)]. Metal-free samples of FurC* (subsequently referred to as apoFurC*) were obtained by dialysing (24 h) the protein (1 ml, 100–250 µM) in 1 litre of sodium acetate buffer (5 mM, pH 5.3; 300 mM NaCl) that contained 1 g of the chelating resin Amberlite IRC-718 to bind metal ions [20]. Dialysis was repeated against Hepes buffer (5 mM, pH 6.5; 300 mM NaCl) that contained fresh chelating resin. For potentiometric titrations, the protein was subsequently dialysed against NaCl (300 mM) that was adjusted to the desired pH.

During purification of FurC*, protein concentration was determined by the Bradford method with BSA as a standard [32]. A molar absorption coefficient (ϵ_{280}) for the FurC* monomer of 2020 M⁻¹ · cm⁻¹ was determined for pure FurC* by the method of Gill and von Hippel [33] on the basis of the UV absorption spectrum of the protein in the presence and absence of guanidinium chloride. Protein concentrations are stated as the concentration of monomer. Protein purity and integrity were assessed by MS with a SCIEX ABI300 triple quadrupole mass spectrometer (PerkinElmer Sciex) equipped with an electrospray source.

Sedimentation velocity analysis

Samples of FurC* [6, 41, 82 and 164 µM in sample buffer (300 mM NaCl and 5 mM Hepes, pH 6.5)] were analysed by sedimentation velocity in the absence and presence of Zn²⁺ (5 molar equivalents). An additional sample (6 µM) was evaluated in the same buffer and in the presence of 1 molar equivalent of Zn²⁺. Data (50 scans) were collected using a Beckman Optima XL-I analytical ultracentrifuge (50 000 rev./min at 20 °C), a four-hole rotor (An-60Ti), Epon double-sector centrepieces (12 mm) and quartz windows. Before each run, the windows and Epon centrepieces were washed with nitric acid to eliminate trace metal ion contamination. Sample buffer was placed into the reference sector of the centrepiece. Radial scans were acquired at 230 or 280 nm with no interval set between scans and radial steps of 0.003 cm in continuous mode. The data were analysed as a $c(s)$ (sedimentation coefficient) distribution of Lamm equation solutions calculated with the program SEDFIT [34]. The regularization parameter p was set at 0.95 (high confidence level), and sedimentation

coefficient increments of 200 were used in the appropriate range for each sample. The $c(M)$ (molar mass distribution) distributions were obtained from the corresponding $c(s)$ distributions with the program SEDFIT, and the value of 1.38 for f/f_0 obtained from the fitted data was used in these transformations.

Spectroscopic characterization

Electronic absorption spectra were obtained with a Cary Model 4000 spectrophotometer equipped with a Peltier temperature controller. Fluorescence spectra were collected with an ISS-K2 spectrofluorimeter. Excitation (275 nm) and emission slit widths were selected to achieve resolutions of 4 and 2 nm respectively. CD measurements were performed with a Jasco J-810 CD spectrometer equipped with a Peltier device for temperature regulation. CD spectra were recorded at a scan rate of 50 nm/min and a bandwidth of 1 nm. Spectra reported here represent the average of four scans. Protein samples (10–50 µM FurC*) were prepared in Hepes buffer (5 mM; 300 mM NaCl). Spectra were recorded at 25 °C, except for thermal stability studies. Analysis of CD spectra for evaluation of secondary structure was performed with the K2D algorithm [35,36].

Potentiometric titrations

The change in proton binding that is associated with the binding of Zn²⁺, Co²⁺, Ni²⁺, Mn²⁺, Cu²⁺ and Fe³⁺ to apoFurC* (5.69–6.44 µM) was studied at 25 °C by the method of Laskowski and Finkenstadt [37] with a Radiometer ABU93 Triburet operated under computer control as described previously [38]. Difficulties related to oxidation of Fe²⁺ by dissolved dioxygen prevented consideration of the binding of this species to FurC*. Radiometer glass (pHG201) and reference (XR130) electrodes were used to measure pH. Atomic absorption metal-ion standards (Zn²⁺, Co²⁺, Mn²⁺, Cu²⁺; Titrisol, EM Science; Ni²⁺, Fe³⁺; VWR) were diluted with NaCl solution (final ionic strength of 0.3 M) for use as titrants. Protein and titrant solutions were flushed extensively with humidified argon to remove dissolved CO₂, and the titrator vessels and solution transfer tubing were washed with 5 mM HCl/0.1 M KCl to remove adsorbed metal ions. Titration curves (40–70 points) indicate the volume of standardized acid or base required to re-establish the starting pH after each addition of metal ion solution and are expressed in terms of molar fraction of exchanged protons, H_e^+ , versus the ratio of [metal ion]/[FurC*]. From these data, association constants (K_A) and the saturating value of H_e^+ , q , were determined by non-linear regression analysis with the program SCIENTIST (version 2.02, MicroMath) as described previously [39].

RESULTS

Expression and purification of the C-terminal metal-ion-binding domain of FurC*

Several DNA constructs were evaluated for expression of the C-terminal fragment (residues 62–134) of *Ps. aeruginosa* Fur. Attempts to express FurC* in *E. coli* BL21(DE3) without an N-terminal tag were unsuccessful. Fusion of FurC* to an MBP (maltose-binding protein) domain, on the other hand, resulted in good expression of soluble protein, but treatment with Factor Xa to release the MBP domain was incomplete and non-specific. Moreover, subsequent purification with an amylose-affinity column was complicated because FurC* appeared to block the maltose-binding site of MBP. An N-terminal poly-His purification tag also resulted in efficient expression, but the His-tagged FurC*

formed inclusion bodies that required stringent treatment for solubilization, and the cleaved FurC* could not be separated easily from uncleaved fusion protein owing to the high native affinity of FurC* for metal ions immobilized on IMAC columns. Other N-terminal sequences that improved expression of other proteins [40] proved unsatisfactory for expression of FurC*.

Ultimately, efficient expression of FurC* in *E. coli* BL21(DE3) was achieved as a fusion construct with the B1 domain of Protein G [30]. From this construct, the apoFurC* domain could be obtained in good yield (10–15 mg/l of culture) following hydrolysis of a thrombin recognition sequence to produce a product of > 95 % purity as evaluated by SDS/PAGE analysis. The mass of the FurC* domain with the N-terminal dipeptide Gly-Ser remaining after removal of the Protein G B1 domain by hydrolysis with thrombin was 8503 a.m.u. as determined by electrospray MS. This value compares well with the predicted mass of 8502 a.m.u., so no unexpected or additional covalent modifications of the protein occurred.

Sedimentation velocity experiments

Self-association of FurC* was evaluated by analytical ultracentrifugation for a range of FurC* concentrations in the presence and absence of Zn^{2+} . The distributions of sedimentation coefficients, $c(s)$, at low and high protein concentration and in the presence and absence of 5 molar equivalents of Zn^{2+} are shown in Figure 3(A). Related results are also provided as a series of $c(M)$ distributions (Figure 3B) that were obtained by transforming $c(s)$ results obtained at low FurC* concentration (6 μM) in the absence and presence of 1 and 5 molar equivalents of Zn^{2+} (Figure 3B). It is clear that in the absence of metal ion, FurC* is dimeric even at high protein concentration. The values for sedimentation coefficient (1.67 S) and molecular mass (~ 17 kDa) obtained from these analyses are those expected for dimeric FurC*. In the presence of 5 molar equivalents of Zn^{2+} , some evidence of formation of larger oligomers is apparent at the highest concentration (164 μM) of FurC* (Figure 3A). Nevertheless, at low protein (6 μM) and metal ion concentration (6 μM), FurC* is fully dimeric (Figure 3B), and at this concentration most of the protein (~ 88 %) is dimeric even in the presence of 5 molar equivalents of Zn^{2+} .

Spectroscopic characterization and structural stability

The CD spectrum of apoFurC* is independent of pH between pH 6 and 7.4 at 15 °C (Figure 4A) and is consistent with a secondary structure for the domain (37 % α -helix and 26 % β -strand) that is the same as that defined for this region of the protein by the crystallographically determined structure of full-length Fur. The temperature dependence of this spectrum at pH 6.5 is shown in Figure 4(B). Based on this information, the thermal stability of FurC* was evaluated by monitoring the change in ellipticity with increasing temperature (Figure 4C). At pH 6.0, for example, the midpoint melting temperature (T_m) is 68 °C, whereas at pH 8.0, the T_m is 39 °C. At most, 90–95 % of the original ellipticity could be recovered by returning the sample to 15 °C after thermal denaturation, indicating that the thermal unfolding is not fully reversible. In addition, the thermal stability of apoFurC* is a function of metal ion binding, as illustrated for the binding of Zn^{2+} (Figure 4D). In the presence of 1 molar equivalent of Zn^{2+} per apoFurC* monomer, significant secondary structure persists up to approx. 90 °C regardless of pH between pH 6 and 8. Increasing $[\text{Zn}^{2+}]$ further affords additional stability, but the magnitude of this effect is difficult to quantify, owing to the inability of our instrumentation to operate at temperatures sufficiently high to observe an endpoint.

Figure 3 Sedimentation velocity analysis of FurC* (all curves are normalized)

(A) $c(s)$ distribution for $[\text{FurC}^*] = 6 \mu\text{M}$ (no Zn^{2+}) (grey line, grey closed circles), $[\text{FurC}^*] = 6 \mu\text{M}$ and $[\text{Zn}^{2+}] = 30 \mu\text{M}$ (black line, open circles), $[\text{FurC}^*] = 164 \mu\text{M}$ (no Zn^{2+}) (light grey line), and $[\text{FurC}^*] = 164 \mu\text{M}$ and $[\text{Zn}^{2+}] = 820 \mu\text{M}$ (black line). Inset: expansion of the region reporting larger oligomers. (B) $c(M)$ distribution for $[\text{FurC}^*] = 6 \mu\text{M}$ (no Zn^{2+}) (grey closed circles), $[\text{FurC}^*] = 6 \mu\text{M}$ and $[\text{Zn}^{2+}] = 6 \mu\text{M}$ (light grey line), and $[\text{FurC}^*] = 6 \mu\text{M}$ and $[\text{Zn}^{2+}] = 30 \mu\text{M}$ (black line, open circles).

Metal ion binding stoichiometry and affinity of FurC*

The affinity of apoFurC* for metal ions was assessed by potentiometric titration. The results of these titrations are illustrated in Figure 5, and the numerical parameters derived from these titrations at pH 6.5 are summarized in (Table 1). The proton release that occurs as the result of titrating apoFurC* with Zn^{2+} at pH 6 (Figure 5A, Δ) is representative of the behaviour observed. In all cases, the simplest model that provides a reasonable fit to the titration results is one in which metal ions bind at two independent sites with inequivalent affinity. The apparent association constants for Zn^{2+} binding that are derived from fitting these data to a two-site model are $1.0(\pm 4) \times 10^7$ and $5.0(\pm 2) \times 10^5 \text{ M}^{-1}$, and binding at these sites is associated with a net release of $2.08(\pm 4)$ and $2.00(\pm 4)$ protons respectively. At pH 6.5 (Figure 5A, $\square$), the association constant for Zn^{2+} binding to the site with greater affinity decreases approx. 2-fold, while the number of protons released upon Zn^{2+} binding increases slightly. On the other hand, the stability of Zn^{2+} binding at the lower-affinity site increases by an order of magnitude, and the number of protons

Figure 4 CD spectra of FurC* as a function of pH, temperature and metal ion binding

(A) Spectra at pH 6.0, 6.5, 7.0, 7.4 (boldface, solid curves) and 8.0 (dashed curve). (B) Spectra as a function of temperature (pH 6.5) in 15 °C intervals starting at 15 °C (the arrow indicates effect of increasing temperature). (C) Thermal stability at pH 6, 6.5, 7, 7.4 and 8.0; the arrow indicates the direction of decreasing pH. (D) Effect of 0, 1, 2 and 4 molar equivalents of Zn^{2+} per FurC* monomer (the $[\text{Zn}^{2+}]/[\text{apoFurC}^*]$ ratios are indicated).

released upon Zn^{2+} binding to this site decreases to 1.00(3) at pH 6.5. At pH 7.39 (Figure 5A, ○), the quality of the fit to the two-site model is not as good as observed in all other cases [$K_a = 5(\pm 4) \times 10^6$ and $K_b = 1.3(\pm 1) \times 10^5 \text{ M}^{-1}$], but this result is probably attributable to difficulties arising from the behaviour of Zn^{2+} at this elevated pH and relatively high chloride concentration. These conditions promote changes in Zn^{2+} co-ordination chemistry related to increased aquo-ion hydrolysis and associated polymerization of aquo- Zn^{2+} species [41]. Similarly, potentiometric titration of apoFurC* with Fe^{3+} or Cu^{2+} was precluded by the aggregation of these metal ions under the conditions required for these experiments.

Potentiometric titrations of apoFurC* with Co^{2+} , Ni^{2+} and Mn^{2+} at pH 6.5 are shown in Figure 5(B). The relatively slow change in pH observed upon addition of Ni^{2+} necessitated at least a doubling of equilibration times between each addition of nickel titrant. Under these conditions, the titration curves obtained on addition of Co^{2+} or Ni^{2+} solution to apoFurC* are similar to those obtained with Zn^{2+} and could be fitted to the same two-site model. However, the parameters obtained with these metal ions differ substantially (Table 1). The affinity of the lower-affinity site for Ni^{2+} , for example, is approx. 8-fold greater than it is for Co^{2+} , which is in turn approx. 5-fold greater than for Zn^{2+} (i.e. $\text{Ni}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+}$). In contrast, metal ion affinity for the high-affinity site varies in the order $\text{Ni}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+}$. The difference in affinities between the low- and high-affinity sites is greater for Zn^{2+} (i.e. ~170-fold) and smaller for Co^{2+} and Ni^{2+} (~10-fold).

Although the titration data obtained with Mn^{2+} are fitted best with a two-site model, the titration results for this metal ion appear

to differ from those obtained for the other metal ions studied, in that the release of protons appears to be nearly proportional to the concentration of metal ion added (Figure 5B, □). This response stems from the significantly poorer binding of Mn^{2+} at both the low- and high-affinity binding sites, which presumably reflects the greater ionic radius of Mn^{2+} or the preference of this metal ion for carboxylate rather than nitrogenous ligands.

Detection of metal ion binding to FurC* by fluorescence spectroscopy

The FurC* construct contains no tryptophan, and of the three tyrosine residues present in the wild-type protein, only the tyrosine near the C-terminus of the domain is retained. This tyrosine residue is 12–15 Å (1 Å = 1 nm) from the crystallographically defined metal-ion-binding sites within a single subunit and approx. 12 Å from the corresponding sites of the neighbouring subunit in the dimer [23]. The fluorescence emission spectrum of apoFurC* observed on excitation at 275 nm is dominated by the emission from this tyrosine residue (Figure 6). In the presence of 1 molar equivalent of Co^{2+} , the emission maximum remains at 305 nm, but the emission intensity decreases substantially (Figure 6A). Less quenching results from the further addition of Co^{2+} , suggesting that this decreased fluorescence is not the result of collisional quenching, but results from non-radiative resonance energy transfer from the tyrosine excited state to neighbouring paramagnetic Co^{2+} ion(s) bound to the protein. The loss in fluorescence can be reversed quantitatively by adding Zn^{2+} (Figure 6B). Zn^{2+} is diamagnetic and, therefore, exhibits little or no quenching of the

Figure 5 Potentiometric titration data and fits obtained for the addition of selected metal ions to the FurC* construct from *Ps. aeruginosa* (25 °C, 300 mM NaCl)

H_e^+ is the net number of protons that were released or absorbed upon addition of a standard solution of metal ion. The ratio of metal ion concentration to protein concentration (abscissa) is expressed in terms of FurC* monomer concentration. Positive values represent proton release. For clarity, only every other data point is shown. (A) Binding of Zn^{2+} at pH 5.97 (Δ), 6.45 ($\square$) and 7.39 ($\circ$). (B) Binding of Ni^{2+} (Δ), Co^{2+} ($\circ$) and Mn^{2+} ($\square$) at pH 6.5.

Table 1 Metal ion affinity exhibited by apoFurC* from *Ps. aeruginosa* as determined by potentiometric titration (pH 6.5, 300 mM NaCl, 25 °C)

Uncertainties (S.D.) of stability constants are provided in parentheses. The stoichiometries of proton release for the two binding sites (q_a and q_b [39]) are also indicated.

Metal ion	K_a (M^{-1})	K_b (M^{-1})	q_a	q_b
Zn^{2+}	$5.7(\pm 3) \times 10^6$	$3.2(\pm 2) \times 10^4$	2.26	1.06
Mn^{2+}	$2.0(\pm 3) \times 10^4$	$1.5(\pm 2) \times 10^3$	0.52	5.6
Ni^{2+}	$1.0(\pm 7) \times 10^7$	$1.3(\pm 2) \times 10^6$	2.1	1.8
Co^{2+}	$2.0(\pm 2) \times 10^6$	$1.76(\pm 1) \times 10^5$	1.78	1.86

fluorescence from nearby fluorophores. In view of the known structure of the protein and the results from potentiometric titrations shown above, it appears most likely that Zn^{2+} displaces Co^{2+} from its binding site(s) near the tyrosine, and relatively unlikely that binding of Zn^{2+} at a different site induces structural changes of the protein that reduce the efficiency of quenching by Co^{2+} .

DISCUSSION

The present results provide experimental evidence to support the proposal arising from the crystallographic structure of *Ps. aeruginosa*

inosa Fur [23] that the C-terminal region of the protein comprises a dimerization domain (FurC*). In addition, the current results provide evidence that this dimerization domain can be expressed as a stable ordered structure that behaves in a manner consistent with that anticipated by the structure of N-terminal domain in the full-length protein. Specifically, the sedimentation velocity experiments demonstrate that the FurC* domain is dimeric in the presence and absence of metal ions. Small amounts of larger oligomers can form as the protein concentration and metal ion concentration increase, a result that may be attributed to the observation of zinc ions unrelated to the structural and sensory metal-ion-binding sites bound on the surface of Fur between dimers in the crystal structure [23]. The far-UV CD spectrum of FurC* indicates that the secondary structure of this domain possesses α -helical and β -sheet contents similar to that of the corresponding C-terminal portion of the full-length protein (Figure 2). The conclusion that FurC* forms a well-ordered discrete structure in solution is further supported by the striking thermal stability of FurC* (Figure 4) and by observation of metal ion binding stoichiometry predicted by the structure of the full-length protein. Clearly, the behaviour of this domain in solution permits at least some physical studies that cannot be undertaken with wild-type Fur, owing to the propensity of the latter protein to aggregate (results not shown). This observation implies that the N-terminal DNA-binding domain is not only responsible for recognition of the Fur-box sequence, but makes a significant contribution to formation of higher-order oligomers. Thus future efforts to design variants of full-length Fur with improved solubility properties (e.g. [42,43]) should logically give priority to modification of the N-terminal domain of the protein. The solubility of the FurC* is, nevertheless, limited, and the conditions of high ionic strength and slightly acidic pH used for the potentiometric titrations were selected because they were the least problematic.

The crystallographically determined structure [23] identifies two principal metal-ion-binding sites in the full-length *Ps. aeruginosa* Fur protein. The site proposed to comprise the sensory binding site for iron (site 1 [23]) involves His⁸⁶, Asp⁸⁸, Glu¹⁰⁷ and His¹²⁷, and the site proposed to stabilize the structure of the protein through binding of Zn^{2+} (site 2 [23]) involves His³², Glu⁸⁰, His⁸⁹ and Glu¹⁰⁰. Although the affinity of the structural metal-ion-binding site for metal ions is expected to be greater than that of the sensory binding site, FurC* lacks His³², one of the residues that co-ordinates the Zn^{2+} ion at the interface between the N- and C-termini of the protein in the structural metal-ion-binding site (Figure 2). Thus the stability of Zn^{2+} binding to this site in the dimerization domain is expected to be significantly less than that of Zn^{2+} binding to the corresponding site in full-length Fur. Based on these considerations, we assign the binding parameters for the lower-affinity metal-ion-binding site summarized in Table 1 to the residual structural Zn^{2+} binding site of FurC*.

The proton release that accompanies binding of bivalent metal cations to the two metal-ion-binding sites of the FurC* domain results from the deprotonation of functional groups that co-ordinate the bound metal ions and from changes in the pK_a of non-coordinating amino acid side chains that occur as the result of metal ion binding. Thus data derived from potentiometric titrations provide highly sensitive and precise measurements of metal ion binding. Although unambiguous quantitative attribution of protons that are released during these titrations to specific titratable groups is not possible without a great deal of additional experimental information, some mechanistic possibilities may be proposed. If the structure of Fur with both metal-ion-binding sites occupied is indicative of the structure of the apo-protein and the FurC* domain, all of the residues identified as comprising the metal-ion-binding sites (see above) are highly protected from

Figure 6 Fluorescence emission spectra of apoFurC* (5 mM Hepes buffer, pH 6.5, and 300 mM NaCl, 25 °C, 275 nm excitation) in the absence (---) and presence (—) of bivalent metal ions

The arrows indicate increasing amounts of (A) Co^{2+} and (B) Zn^{2+} . (A) Spectra collected after the addition of 1 molar equivalent per FurC* monomer of Co^{2+} (thick trace) followed by 2 and 4 molar equivalents (thin traces) of Co^{2+} . (B) Spectra of FurC* containing 4 molar equivalents of Co^{2+} with 1 and 2 molar equivalents of Zn^{2+} (thin traces) and 4 molar equivalents of Zn^{2+} (thick line). a.u., arbitrary units.

the solvent. In this case, the neutral states of these groups should be favoured in the absence of bound metal ions such that the acidic ligands are likely to exhibit relatively high pK_a values, and the imidazole ligands are likely to exhibit relatively low pK_a values. Thus most of the proton release observed on binding of metal ions would result from deprotonation of Asp⁸⁸ and Glu¹⁰⁷ (at site 1) and from Glu⁸⁰ and Glu¹⁰⁰ (at site 2). These electrostatic characteristics of the sensory metal-ion-binding site and the anticipated charge neutrality of the occupied metal-ion-binding sites are presumably related to the relative stability of the Fur- Fe^{2+} complex and to the observation that the reduction potential of iron bound to this site is relatively high [18,23]. While characterization of the electrostatic properties of Fur is essential to comprehensive understanding of the mechanism by which this protein normally functions, such understanding may also have significant practical implications with regard to the Fur homologue from *H. pylori*. The survival of this pathogen in an environment of low pH has been clearly linked to the role of the *H. pylori* Fur protein both as an iron metallo-regulatory protein and as a pH-sensor [12,13].

Notably, the relative affinities for the high-affinity (sensory) site exhibited by the metal ions studied here follow the Irving-Williams order [44], while for the low-affinity (modified structural) site, the stability of Zn^{2+} binding is greater than this relationship predicts. These observations lead us to conclude that the co-ordination environment of the sensory site provided by FurC* to each of these bivalent metal ions is the same, while the co-ordination environment of Zn^{2+} at the modified structural site differs from that of the other metal ions, possibly through recruitment of an additional protein ligand. The fact that Zn^{2+} , Co^{2+} and Ni^{2+} all bind with reasonable affinity to the sensory metal-ion-binding site is consistent with the observation that all of these metal ions trigger *Ps. aeruginosa* Fur binding to iron-box DNA *in vitro* [21]. In view of the similarity of the co-ordination chemistry of Mn^{2+} and Fe^{2+} and the fact that Mn^{2+} is often used as an analogue of iron in gel-shift assays, the lower affinity of Mn^{2+} observed in the present study is of particular interest and bears further analysis in studies of the native protein in the presence and absence of an oligonucleotide bearing the sequence of the Fur-box.

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