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A ZnS_4 Structural Zinc Site in the *Helicobacter pylori* Ferric Uptake Regulator

Sylvia Vitale,[‡,§] Caroline Fauquant,[‡,§,∥] David Lascoux,[⊥] Kristine Schauer,[#] Christine Saint-Pierre,[△] and Isabelle Michaud-Soret*[,§]

[§]*CNRS UMR 5249 Laboratoire de Chimie et Biologie des Métaux, Commissariat à l'Energie Atomique (CEA), Direction des Sciences du Vivant (DSV), l'Institut de Recherches en Technologies et Sciences pour le Vivant (iRTSV), and Université Joseph Fourier, 17 rue des Martyrs, F-38054 Grenoble Cedex 9, France,* [⊥]*Laboratoire de Spectrométrie de Masse des Protéines, Institut de Biologie Structurale, Jean-Pierre Ebel (UMR 5075 CNRS/CEA/UJF), F-38027 Grenoble Cedex 1, France,* [△]*Laboratoire des Lésions des Acides Nucléiques, DSM/INAC/Service de Chimie Inorganique et Biologique, UMR E-3 CEA/UJF CNRS FRE 3200, 17 rue des Martyrs, Grenoble F-38054 Cedex 9, France, and* [#]*Unité Pathogenèse de Helicobacter, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France* [‡] *These authors contributed equally to this work and have made substantial contributions to the work.* [∥]*Current address: Laboratoire d'Enzymologie et Biochimie Structurales (LEBS), CNRS, Bât. 34, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette, France*

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ABSTRACT: The ferric uptake regulator, Fur, is a global bacterial transcriptional regulator using iron as a cofactor to bind to specific DNA sequences. This paper describes the biochemical characterization of the native ferric uptake regulator from *Helicobacter pylori* (HpFur): oligomeric state, metal content, and characterization of a structural metal-binding site. HpFur contains six cysteines with two CxxC motifs, which makes it closer to *Bacillus subtilis* PerR (BsPerR) than to *Escherichia coli* Fur (EcFur). Chemical modifications of cysteine residues using iodoacetamide followed by mass spectrometry after enzymatic digestion strongly suggest that these two CxxC motifs containing cysteines 102−105 and 142−145 are involved in zinc binding in a ZnS_4 metal site. The other two cysteines (78 and 150) are not essential for DNA binding activity and do not perturb metal binding as demonstrated with the characterization of a FurC78SC150S double mutant. Chelating agent such as EDTA disrupts the dimeric structure into monomer which did not contain zinc anymore. Reconstitution of dimer from monomer requires reduction and Zn^{2+} binding. Cadmium(II) substitution allows also dimer formation from monomer, and Cd(II)-substituted FurC78SC150S mutant presents a characteristic absorption of a $Cd(II)Cys_4$ metal-binding site. These results establish that coordination of the zinc ion in HpFur is $ZnCys_4$, therefore closer to the zinc site in BsPerR than in EcFur. Furthermore, the redox state of the cysteines and the zinc binding are essential to hold the *H. pylori* Fur in a dimeric state.

Analysis of adaptive responses in *Heliobacter pylori* is particularly interesting since this pathogen persistently colonizes half of the human population and can cause chronic gastritis and gastroduodenal ulcers as well as cancers which had also been linked to iron deficiency anemia (*1*). *H. pylori* has developed unique strategies to survive severe acidic shocks (pH as low as 1.4) and multiply in the hostile gastric environment at moderately acidic pH (around 5) (*2*). Three transcriptional regulators were found to be central in the response of *H. pylori* to acidity: (i) the iron responsive regulator HpFur[1] (*3*−*6*), (ii) the nickel responsive regulator HpNikR (*7*), and (iii) the ArsRS genes encoding a two-component regulatory system (*8*). An original overlap between the nickel, iron, and acid-regulated genes was revealed, including autoregulation of the genes encoding these two metalloregulators, Fur and NikR (*4*, *9*, *10*). Both HpNikR and HpFur were found to be involved in nickel and iron homeostasis, respectively, and to be required for efficient colonization of the mouse gastric mucosa by *H. pylori* (*4*, *5*). HpNikR and HpFur were shown not only to repress the transcription of several genes but also to directly upregulate the expression of others such as ferritin (Pfr) (*11*−*13*). The molecular mechanisms underlying this newly described activation property are unknown.

At a cellular level, HpFur has been shown to play an intricate role in adaptation of the bacterium to two conditions known to oscillate within the gastric mucosa: iron limitation and low pH. The Fur regulon of *H. pylori* consists of 59 genes directly

*Corresponding author. E-mail: imichaud@cea.fr. Phone: +33 4 38 78 99 40. Fax: + 33 4 38 78 54 87.

[1]Abbreviations: HpFur, ferric uptake regulator from *Helicobacter pylori*; PaFur, ferric uptake regulator from *Pseudomonas aeruginosa*; MtZur, zinc uptake regulator from *Mycobacterium tuberculosis*; BsPerR, PerR for *Bacillus subtilis*; EcFur, ferric uptake regulator from *Escherichia coli*; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; ICP-AES, inductively coupled plasma−atomic emission spectrometry; ICT, trypsin−chymotrypsin inhibitor; IPTG, isopropyl β-D-1-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAR, 4-(2-pyridylazo)resorcinol; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid.

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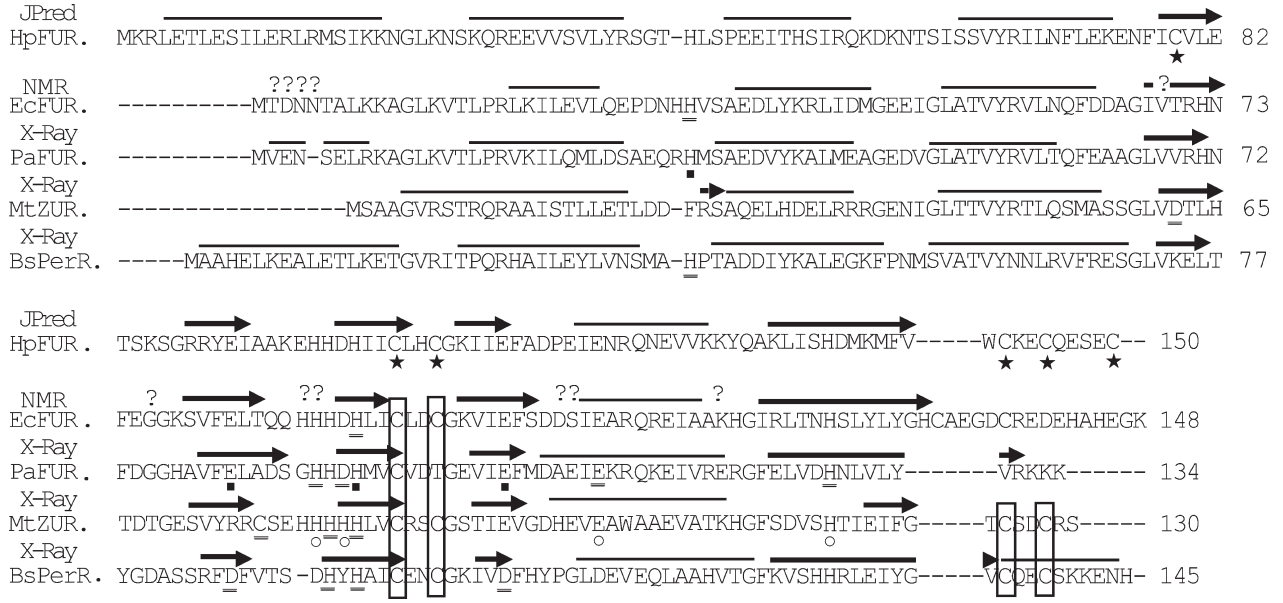


FIGURE 1: Sequence alignment of *H. pylori* Fur with Fur from *P. aeruginosa* and *E. coli*, Zur from *M. tuberculosis*, and PerR from *B. subtilis* and secondary structure comparison. Experimentally (NMR or X-ray) or predicted determined secondary structure elements are indicated with an arrow for β-strands and a line for α-helices, and a ? corresponds to unassigned residues in *E. coli* Fur (*23*). Metal-binding ligands described in the literature (*39*) are noted with a square (■) for the structural zinc site (S1), underlined for the regulatory iron site (S2), and noted with a circle (○) for another metal-binding site (S3). Cysteines which are involved in a metal-binding site and HpFur cysteines are noted with a star (★).

regulated, 25 of which are positively regulated (*12*). Many of these genes, including *nikR*, have been previously annotated as acidic stress associated. Introduction of a Fur mutation causes aberrant expression of a large number of genes and decreases the ability of *H. pylori* to colonize an animal model (*4*, *5*). It shows the importance of the integrity of HpFur for the virulence and its potential interest as an antibacterial target. In addition, it has been suggested that the upregulation of some genes does not require metal-activated Fur protein and involves Fur polymerization along the target DNA (*13*, *14*). A hypothetical mechanism was proposed by Delany et al. (*14*) but needs to be tested with native proteins and controlled metal concentration systems.

The *fur* gene from *H. pylori* was previously identified (*15*), and the deduced protein sequence (HpFur) corresponds to a protein of 17681 Da containing 150 amino acids which had a high sequence homology with other Fur proteins (59−49% similarity and 37−23% identity). HpFur shares 32.4%, 28.9% identities and 66.9%, 70.3% similarities with Fur from *Escherichia coli* (EcFur) and *Pseudomonas aeruginosa* (PaFur) and also 24% and 26.4% identities with *Bacillus subtilis* PerR and *Mycobacterium tuberculosis* Zur, respectively, both Fur-like proteins with recently solved X-ray structures (from SCANPS, http://www.ebi.ac.uk/Tools/). Figure 1 describes the alignment of these sequences.

The first X-ray structure of a Fur protein (from *P. aeruginosa*) has been reported in a zinc-substituted form (*16*). The structure shows that the protein is a homodimer containing a winged helix−turn−helix motif (WHTH) and a dimerization domain. As expected from previous extensive spectroscopic studies of EcFur (*17*−*19*), two relevant metal-binding sites were identified in the crystal structure: a regulatory site (in the dimerization domain) responsible for activation of the DNA binding activity and a structural zinc site(*18*, *20*, *21*). The structural site in PaFur contains a zinc ion in a tetrahedral geometry coordinated by two histidines (His32 and His89) and two glutamates (Glu80 and Glu100). In contrast, for EcFur, X-ray absorption studies showed that the zinc ion is coordinated by two cysteines and one or two aspartates or one histidine and one aspartate (*18*). The two cysteines, Cys92 and Cys95, have been identified as ligands of the zinc atom and are essential to the dimerization (*21*−*23*). These two cysteines are conserved in a large number of Fur proteins such as EcFur and HpFur, MtZur, and BsPerR, but only one is conserved in PaFur. In addition to this CxxC conserved motif, another set of conserved cysteines exist in the C-terminal part of the Fur and Fur-like proteins corresponding either to CxxC such as in HpFur, MtZur, or BsPerR, for example, or to a CX_4C motif such as in EcFur. MtZur- and BsPerR-resolved structures described a $ZnCys_4$ structural zinc site, and we can suspect that HpFur may contain the same type of coordination. The presence of the conserved CxxC cysteine motifs seems not a proof of their involvement in the binding of structural zinc ion. Indeed, it has been recently described that Cys 92 and C95 from *Vibrio harveyi* Fur were not essential (*24*), contrarily to the corresponding one from *Edwarsiella tarda* Fur (*25*). This makes more complex the variability of the structural zinc sites inside the Fur family (*26*). HpFur contains six cysteines, and it was interesting to identify how many and which of them were bound to a zinc ion.

Furthermore, HpFur is able to partially complement the Δfur *E. coli* strain and cross-reacts with EcFur-directed antibodies (*15*, *27*). This strongly suggests functional and structural homologies between the two proteins. However, biochemical characterization of HpFur had not been described to date. The published data on HpFur considered the mechanism of gene transcription regulation (*3*, *5*, *6*, *10*, *12*−*14*, *28*−*36*). They have been mostly studied using His-tagged proteins (N- or C-terminal tagged) (*10*, *15*, *27*). *Hpfur* have been proposed to be autoregulated in a complex manner involving both the apo form and metal-bound form of the protein (*10*, *14*).

This paper describes the properties of native HpFur: oligomeric state, metal content, and characterization of the zinc binding properties using chemical modification coupled to mass spectrometry analysis. Our data confirm the involvement of four cysteines in zinc binding and identify them within the two motifs

CxxC present in the protein. It establishes that coordination of the zinc ion in HpFur is $ZnCys_4$ and then more comparable to the one in BsPerR or MtZur than the one in EcFur.

MATERIALS AND METHODS

Chemicals. Trizma, Bis-Tris propane, MOPS, and HEPES buffers, EDTA, $ZnSO_4$, $MgSO_4$, trypsin, Lys-C endoproteinase, $NiSO_4$, or $NiCl_2$ were purchased from Sigma-Aldrich.

Cloning and Site-Directed Mutagenesis. The *H. pylori fur* gene (hp1027) was amplified from the genome of the strain *H. pylori* 26695 with primers fur5′-GGAATTCCATATGAAAA-GATTAGAAACTTTGGAATCC and fur3′-GCGGATCCT-TAACATTCACTCTCTTGGCATTC (restriction site underlined). The *Nde*I/*Bam*HI-digested PCR product was inserted into the expression vector pET11a (Novagen), resulting in p*fur3*. This construct was used to produce one plasmid, p*fur2M*, where cysteines 78 and 150 were mutated to serine. The QuikChange site-directed mutagenesis kit (Stratagen) was used for mutagenesis, with the following primers (mutated base underlined): 78fur 5′-CTTAGAAAAAGAAAATTTTATCAGT-GTTTTAGAAACTTCAAAAAGCGGTCG-3′, primer 78bfur 5′-CGACCGCTTTTTGAAGTTTCTAAAACACTGATAA-AATTTTCTTTTTCTAAG-3′, primer 150fur 5′-GCCAAGA-GAGTGAAAGTTAAGGATCCGGCTGC-3′, and primer 150bfur 5′-GCAGCCGGATCCTTAACTTTCACTCTCTT-GGC-3′. The resulting plasmid was sequenced by Cogenics (Meylan, France).

Protein Overexpression and Purification. All of the purification procedures were performed at 4 °C using Amersham Biosciences columns, and centrifugations were run at 6 °C. Wild-type and mutant HpFur proteins were overexpressed in BL21 (DE3) competent cells freshly transformed with recombinant plasmid. Overnight cultures of *E. coli* BL21(DE3) with p*fur3 or* p*fur2M* were diluted 100-fold in 3 L flasks containing 1 L of LB medium supplemented with ampicillin ($150\ \mu g \cdot mL^{-1}$) and grown at 37 °C. Cells were induced with 0.5 mM IPTG for 2 h when OD_{600} reached 0.7. The cells were harvested by centrifugation at 6000*g* for 10 min, resuspended immediately in sonication buffer (20 mM HEPES, pH 7.4, containing 500 mM NaCl, $10\ \mu g \cdot mL^{-1}$ ICT, $4\ \mu g \cdot mL^{-1}$ pepstatin A, and $240\ \mu g \cdot mL^{-1}$ PMSF), and lysed by sonication on ice. The lysate was centrifuged at 20000*g* for 20 min, and then the supernatant at which 24 μg of DNase I (Sigma) was added was dialyzed twice against 2 L of 20 mM HEPES, pH 7.4, 100 mM NaCl, and 3 mM $MgSO_4$ at 4 °C. After the second dialysis, 3 mM EDTA was added to the supernatant which was then centrifuged at 20000*g* for 20 min. The crude protein mixture was loaded on a SP-Sepharose column (HP Hiload 16/10; GE Healthcare) equilibrated with buffer A (20 mM HEPES, pH 7.4, 100 mM NaCl) and 12% buffer B (20 mM HEPES, pH 7.4, 1 M NaCl). HpFur was eluted with a linear gradient ranging from 12% to 100% buffer B with a flow rate of 2 mL/min. After concentration by ultrafiltration (15 mL, 5 kDa cutoff; Millipore) of HpFur fractions, the protein was further purified on a gel filtration column (Superdex 75 prep grade HR16/60; GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.4, 400 mM NaCl, and 5 mM DTT.

Homogeneous HpFur WT samples were obtained, and the purity was verified by SDS−PAGE and mass spectrometry analysis. Pure protein fractions were concentrated (4 mL, 5 kDa cutoff; Millipore) (Supporting Information Figure S1). Protein concentration was determined by UV/vis spectroscopy at 280 nm and the theoretical absorption coefficient of 11460 $M^{-1} \cdot cm^{-1}$ and 11290 $M^{-1} \cdot cm^{-1}$ for the WT and mutant, respectively (http://expasy.org/tools/protparam.html). Approximately 10 mg of pure protein was obtained per liter of bacterial culture. All proteins concentrations are expressed in monomer units.

Metal Quantification. (A) Colorimetric Analysis of Transition Metals with 4-(2-Pyridylazo)resorcinol. The presence of transition metals in purified HpFur proteins was determined as followed. Protein (20 μM) was precipitated with 10% trichloroacetic acid (TCA) for 30 min in ice, and the precipitate was spun down (15 min, 13000 rpm). The supernatant was neutralized with 2 M NaOH and buffered with 1 M Tris-HCl (pH 7.5 at 20 °C) to a final concentration of 0.5 M. The concentration of transition metals in the solution was determined colorimetrically using PAR (4-(2-pyridylazo)resorcinol) (*37*). Samples obtained as described above were diluted five times in buffer containing 100 μM freshly prepared PAR and incubated for exactly 1 h. The change in the UV/vis spectrum of PAR at 495 nm due to binding of transition metals was followed and compared to calibration curves recorded the same day with different transition metals.

(B) Atomic Absorption Spectrometry. The amount of zinc contained in the purified HpFur WT and mutant proteins was determined by flame atomic absorption spectrometry (Perkin-Elmer 560 Norwalk) using an external calibration curve. Prior to analysis, all of the samples (30−50 μM) were buffer exchanged into 20 mM Tris-HCl, pH 7.4, by using NAP-5 columns (GE Healthcare).

Circular Dichroism Spectroscopy. Far-UV CD spectra (195−250 nm) of the reduced dimer apo of EcFur, WT, and the C78SC150S mutant of HpFur were recorded on a Chirascan circular dichroism spectrometer (Applied Photophysics) at 25 °C in a buffer consisting of 20 mM MOPS, pH 7, and 40 mM KCl. A 1 mm path length cell was used for the measurement, and the parameters were set as follows: bandwidth, 1 nm; step resolution, 1 nm; scan speed, 50 nm/min; and response time, 1 s. Each spectrum was obtained as the average of four scans. The protein concentration was typically around 10 μM. Prior to the calculation of the mean residue molar ellipticity, all of the spectra were corrected by subtracting buffer contributions.

Cadmium Binding of HpFur Followed by UV/Vis Spectroscopy. UV/vis spectra were acquired using a Hewlett-Packard 8453 diode array spectrophotometer, thermostated at 20 °C. Cd(II) binding experiments were performed in a 180 μL quartz cuvette (1 cm path length) using 20 μM HpFur WT or HpFur C78SC150S in 20 mM HEPES, pH 7.4, and 500 mM NaCl and adding small volumes of $CdSO_4$.

Analysis of Oligomeric Forms by Size Exclusion Chromatography. Analytical gel filtration chromatography (exclusion chromatography) experiments were performed using an analytical Superdex 75 HR 10/30 column (GE Healthcare), coupled to an Akta purifier chromatography system (GE Healthcare). The experiments were performed at room temperature at 1.0 mL/min flow rate. The column was equilibrated in 20 mM MOPS, pH 7, and 400 mM KCl and calibrated using the LMW gel filtration calibration kit (GE Healthcare) which contains the following mass markers: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and blue dextran 2000 (2 MDa). WT HpFur (100 μL) at 25 μM in the equilibration buffer was loaded on the column.

(A) Dimer to Monomer Conversion. The HpFur-reduced dimer at 25 μM in 20 mM HEPES, pH 7.4, and 500 mM NaCl was incubated in presence of 12.5 mM (500 equiv) EDTA, pH 7.4, at 4 °C during 24 h before being loaded to the Superdex 75 column equilibrated with the same buffer. Fractions containing monomer were collected and concentrated using Ultrafree (15 mL, 5 kDa cutoff; Millipore). The metal concentration in the monomer was determined by the PAR method described above.

(B) Monomer to Dimer Conversion. The HpFur monomer at 70 μM in 20 mM HEPES, pH 7.4, and 500 mM NaCl was incubated in the presence of 1 equiv per HpFur subunit of DTT and 1 equiv per HpFur subunit of $CdSO_4$ or $ZnSO_4$ (not shown) at 25 °C for 15 min before being loaded to the Superdex 75 column equilibrated with the same buffer. Fractions containing dimer were collected. After concentration, the metal concentration in the dimer was determined by the PAR method described above.

Nuclease Protection Assay. HpFur binding to a HpFur box in the *nikR-exbB* intergenic region was assayed by a nuclease protection assay (*38*). Here, the protection of three engineered *Hin*fI, *Eco*RI, and *Ssp*I sites in this region. To obtain the DNA construct, the intergenic region was amplified from the genome of the strain *H. pylori* 26695 with the primers (extension base underlined) P1 5′-AAGCTTGTCTTTATTGGGTGTATCCAT-3′ and P2 5′-GGATCCCTCCTTGTCTATGATAAAAC-3′. The *Hin*dIII/*Bam*HI-digested PCR product was cloned into the vector pUC18 (Fermentas), resulting in p*IGR1*. The *Hin*fI, *Eco*RI, and *Ssp*I restriction sites were introduced in the intergenic region by changing the region GATTG into GATTC, CAATTA into GAATTC, and AATATA into AATATT, respectively, by using the QuikChange site-directed mutagenesis kit. The resulting plasmid, pHP2G, was thus 3003 bp long. The HpFur binding on the furS2 operator site was analyzed with the digestion of the plasmid with *Hin*fI. The HpFur binding on the nikRS2 operator site was analyzed with the digestion of the plasmid with *Ssp*I.

The 10 μL reaction mixture contained 5 μM HpFur and 400 ng of plasmid pHP2G (20 nM final concentration) in the binding buffer consisting of 20 mM Bis-Tris-propane, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, and 10 μM of Co(II). The mixture was incubated for 1 h at room temperature. An additional incubation of 1 h at 37 °C was then carried out in the presence of two units of enzyme (*Hin*fI or *Ssp*I). After a heat inactivation of the enzyme, the reaction mixture was treated with trypsin (Sigma) at 0.25 μM during 2 h at 37 °C. After that, it was mixed with 2 μL of sample loading solution (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol). Immediately afterward, samples were loaded on a 0.8% agarose gel containing ethidium bromide, and the electrophoresis was run at 100 V for 40 min.

Alkylation of the Fur Protein. HpFur (wild type and the C78SC150S double mutant) was taken from stock solutions (respectively 590 and 370 μM) in 20 mM HEPES, pH 7.4, 400 mM NaCl, 1 mM DTT, and 10% v/v glycerol. Proteins were buffer exchanged into 100 mM Tris-HCl, pH 7.5, and 100 mM NaCl with or without 50 mM EDTA at room temperature (25 °C). Buffer exchange was performed using Ultrafree (500 μL, 5 kDa cutoff; Millipore) by dilution/concentration cycles. Proteins were diluted to a final concentration of 50 μM in the appropriate buffer (with or without EDTA). A solution of iodoacetamide was freshly prepared in the same buffer to a final concentration of 3 mM. The alkylation reaction was carried out in the dark. Several concentrations of iodoacetamide had been previously tested (1.5, 0.9, 0.6, and 0.3 mM, corresponding to 5, 3, 2, and 1 equiv of alkylating reagent against the protein). Aliquots of 20 μL (1 nmol of protein) were taken at various times and immediately frozen in liquid nitrogen to stop the reaction.

Enzymatic Digestion of Alkylated HpFur with Endoproteinase Lys-C. Alkylated samples of Fur were buffer exchanged into 100 mM Tris-HCl, pH 7.5, and 100 mM NaCl in order to eliminate iodoacetamide and so prevent the alkylating reaction to continue during enzymatic digestion. Buffer exchange was performed using Ultrafree (500 μL, 5 kDa cutoff; Millipore) by dilution/concentration cycles. The protein was diluted in the same buffer to a final concentration of 5 μM, and about 1% w/w endoproteinase Lys-C was added. The mixture was incubated overnight at room temperature for digestion.

MALDI Mass Spectrometry Analysis. Proteins were diluted into the matrix solvent (50/50/0.2 water/acetonitrile/formic acid) to have a 1 μM solution. Then 0.5 μL was dried with 0.5 μL of matrix solution (sinapinic acid). MALDI-TOF mass spectra were recorded on an Autoflex (Bruker, Bremen, Germany) mass spectrometer in the linear mode. To identify the fast reacting cysteines, the peptides obtained by enzymatic cleavage of the sample alkylated were diluted into the matrix solvent (50/50/0.2 water/acetonitrile/formic acid) to have a 0.5 μM solution. Then 0.5 μL was dried with 0.5 μL of matrix solution (α-cyano-4-hydroxycinnamic acid). MALDI-TOF mass spectra were recorded on an Autoflex (Bruker, Bremen, Germany) mass spectrometer in the reflectron mode.

Structural Model of Native H. pylori Fur. A dimer of HpFur was modeled on the *B. subtilis* PerR structure (PDB code 2FE3) (*39*) using Modeler (*40*) after alignment with Clustal W (*41*). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH Grant P41 RR-01081) (*42*).

RESULTS

It can be predicted from sequence alignments (Figure 1) that four of the six cysteines of HpFur may be involved in zinc binding. Our objectives in this study were to confirm that only four cysteines were bound to the zinc ion and to identify them within the six cysteines of the Fur subunit.

Overexpression and Purification of HpFur and Biochemical Characterization. HpFur is a basic protein (theoretical pI = 8.5) compared to the EcFur homologue (theoretical pI = 5.6), and a purification protocol similar to the one proposed by Alamuri et al. (*11*) was then necessary. The HpFur protein has been purified until homogeneity (SDS−PAGE profile in Supporting Information Figure S1) using SP-Sepharose followed by a gel exclusion purification step on Superdex 75. The N-terminal sequence of the purified protein corresponds to the expected sequence. By using gel exclusion chromatography, the apparent molecular mass of WT HpFur was estimated to 37 kDa based on its elution volume at 10.6 mL at pH 7 (theoretical molecular mass 35.4 kDa). The purified protein (wild type or mutant) appears to exist mainly as a dimer ($HpFur_D$) in solution (Figure 2A), and it was confirmed by mass spectrometry measurements performed under soft nondenaturing conditions. The native dimer contains 0.85 transition metal ion spectroscopically silent/monomer (PAR complexation analysis). ICP-AES analysis demonstrates that $HpFur_D$ contains one zinc/subunit. Contrarily to what was observed for EcFur, no monomeric species was observed during

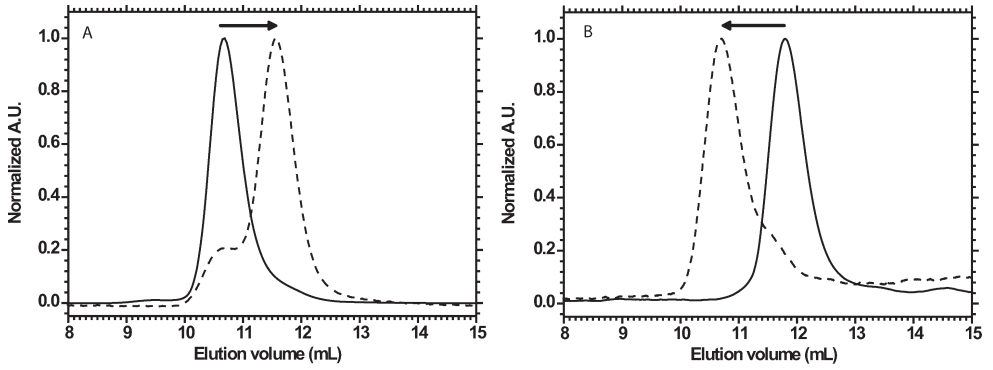


FIGURE 2: Evolution of the oligomeric state of HpFur. (A) Shift of the size exclusion chromatography profile of the dimeric (solid line) to the monomeric (dashed line) species at 25 μM in 20 mM HEPES, pH 7.4, and 500 mM NaCl at 25 °C after an incubation of 24 h at 4 °C in presence of 500 equiv/monomer of EDTA. (B) Shift of the size exclusion chromatography profile of the monomeric (solid line) to the dimeric (dashed line) species at 70 μM in 20 mM HEPES, pH 7.4, and 500 mM NaCl after an incubation of the C78SC150S double mutant during 15 min at 25 °C in the presence of 1 equiv/monomer of DTT and $CdSO_4$. Elution volumes of the dimeric and monomeric species are respectively 10.6 and 11.6 mL. The same data were obtained for the WT protein.

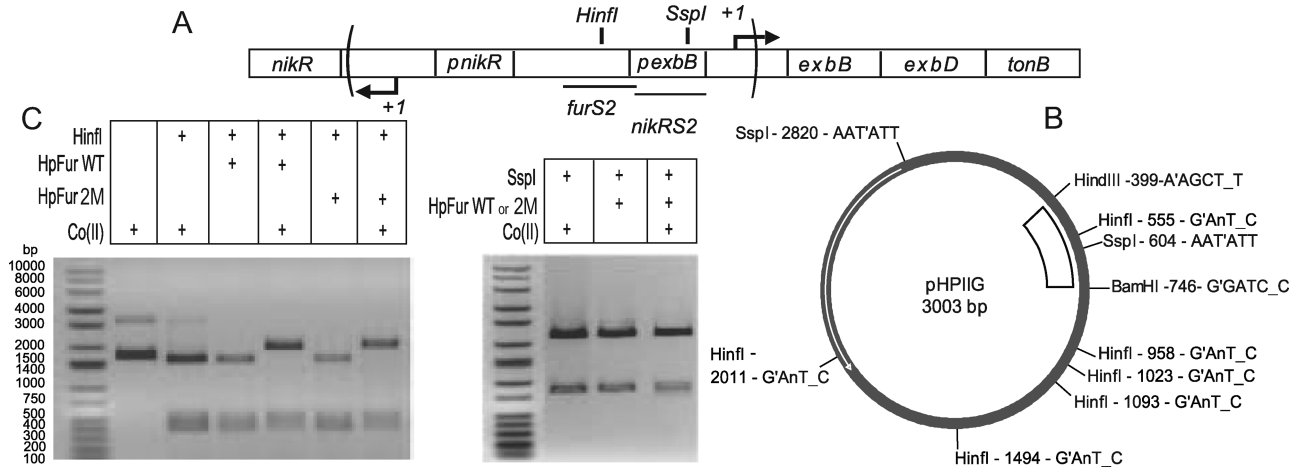


FIGURE 3: HpFur DNA binding to the intergenic region "*nikr-exbB*" followed by a nuclease test. (A) Schematic representation of the intergenic region "*nikR-exbB*" where pnikR, pexb promoters, and transcription start points are specified. Bound regions by HpNikR and HpFur are underlined (*29*), and engineered restriction sites are specified (*Hin*fI in the fur box noted furS2 and *Ssp*I in the nikR box noted nikRS2). (B) The map of the pHPIIG plasmid which contains mutated intergenic regions is specified. (C) Nuclease tests showing the specific and metal-dependent binding of HpFur WT and the C78SC150S double mutant (2M in the figure) on the fur box. Twenty nanomolar plasmid pHPIIG was cleaved by *Hin*fI in the absence (lane 2) or presence of 5 μM HpFuR WT and 2M (apo, lanes 3/5, and active, lanes 4/6). The same experiment was realized with *Ssp*I: absence of HpFur (lane 7) and presence of HpFur WT (apo, lane 8, and active, lane 9). Reaction mixtures were analyzed on 0.8% agarose gel electrophoresis. HpFur binding is observed on the furS2 site (loss of 1547 bp fragment in lanes 4/6 and presence of 1950 bp fragment). No HpFur binding is observed on the nikRS2 site (presence of 2216 and 787 bp fragments in lanes 8 and 9).

the purification process of HpFur, suggesting a higher stability of the dimer. Nevertheless, after a treatment of $HpFur_D$ with a large excess of EDTA overnight in the presence of dithiothreitol as reductant, a shift of its elution volume to 11.6 mL is observed, and it corresponds to a monomeric species ($HpFur_M$) with an apparent molecular mass of 24 kDa. This form contains 0.1 transition metal ion/monomer (ICP-AES analysis). Addition of 1 equiv of cadmium (Figure 2B) or zinc (not shown) per monomer induces a shift from the monomeric to the dimeric species. These results indicate that native HpFur contains one zinc/monomer. This zinc ion is bound in one site noted "S1" involved in the dimerization process. Addition of another 1 equiv of metal/monomer does not have any effect on the oligomeric state (not shown). Nevertheless, we observed oligomerization of the protein at higher pH (pH 8 > pH 7) in the absence of reductant such as dithiothreitol or after H_2O_2 treatment, suggesting that the oligomerization was oxidation dependent. We also observed more oligomerization with the WT protein than with the C78SC150S double mutant, probably due to the poorer stability of the WT monomer. The UV/visible spectra of the native dimer or monomeric forms have no signature of the presence of metal (Zn(II) is spectroscopically silent).

Metal Effect on the HpFur DNA Binding Ability. The DNA binding ability of $HpFur_D$ containing a stoichiometric amount of zinc per subunit as quantified was assayed using a nuclease protection assay as the one described for EcFur binding assay (*38*) . The capacity to protect from digestion was assayed in function of the absence of metal traces or in the presence of Co(II) (used in place of Fe(II) ion which is too easily oxidized) (Figure 3). We introduced *Hin*fI and *Ssp*I sites in the protected region that HpFur and HpNikR bind respectively in the intergenic region *nikr-exbB* (*29*). No protection was observed when the wild-type dimeric protein at 5 μM was tested in the absence of metal traces. The addition of 2 equiv of metal/Fur subunit led to a complete protection of the furS2 site. In parallel, no protection of nikRS2 was observed. It means that HpFur DNA binding on this sequence is specific and metal dependent.

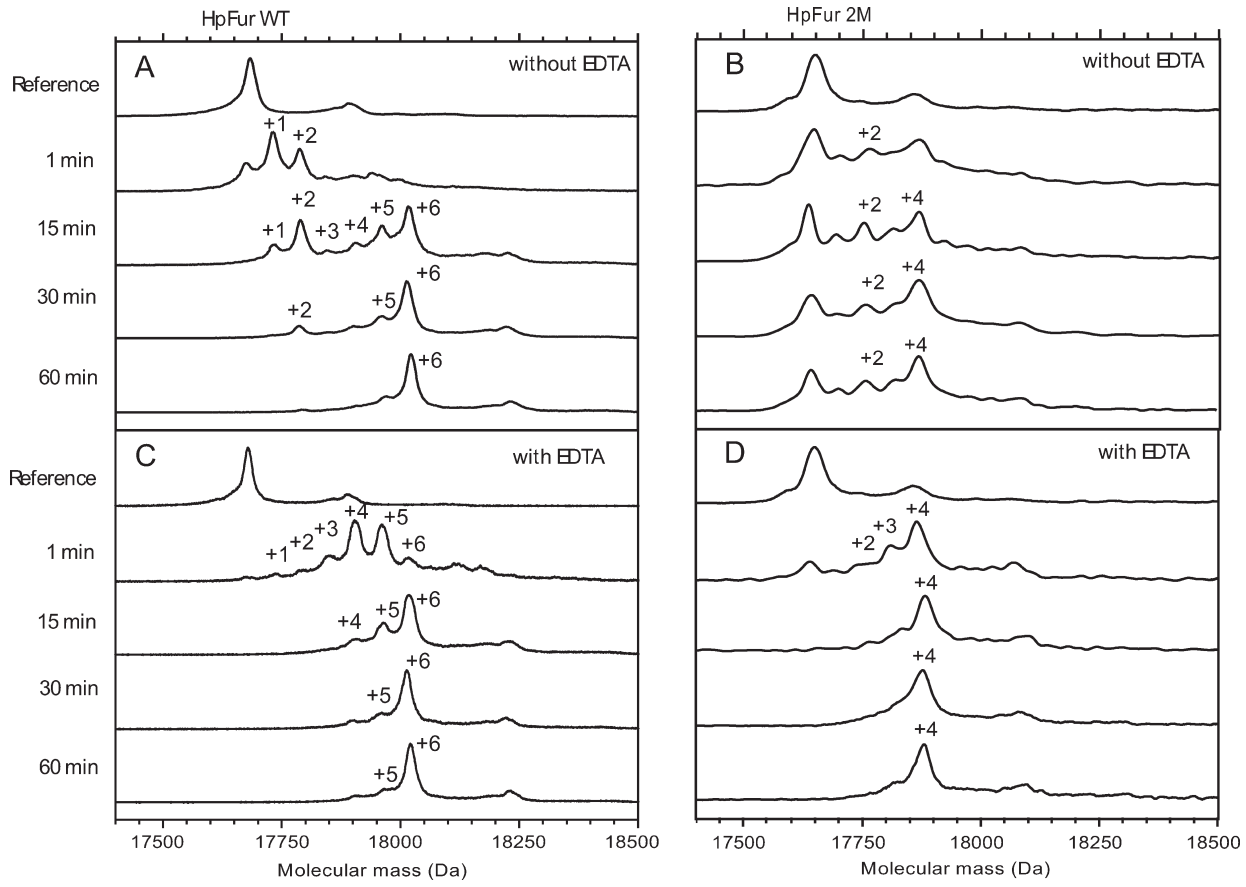


FIGURE 4: MALDI spectra of the kinetics of cysteine alkylation of HpFur WT and the C78SS150S double mutant after iodoacetamide treatment, with or without EDTA. HpFur WT (panels A and C) and the C78SS150S double mutant (2M in the figure; panels B and D) dimers at 50 μM into 100 mM Tris-HCl, pH 7.5, and 100 mM NaCl without (A and B) and with EDTA (50 mM) (panels C and D) were treated with iodoacetamide (3 mM) during various incubation times and analyzed by MALDI-TOF mass spectrometry. The numbers on the figure correspond to the number of alkyl groups covalently linked to the protein.

Identification of the Cysteines Bound to the Zinc Ion. The protein was treated under nondenaturing conditions with iodoacetamide, and the progressive alkylation of the thiol groups was monitored by quenching the reaction at different times and measuring the extent of alkylation by mass spectrometry. Complementary experiments were carried out in the absence or the presence of EDTA, a strong zinc chelator, to determine which of the cysteines were protected from alkylation by the zinc atom. Enzymatic digestion of the modified protein and analysis of the peptide mixture by mass spectrometry enabled fast identification of reactive and protected thiol groups.

Iodoacetamide was used to probe the accessibility of cysteines in HpFur through modification of thiol groups (*21*). As the ionizable side chains (from lysine, arginine, or histidine residues) are generally not involved in these modifications, we considered the addition of alkylating groups to have only a minor influence on the ionization efficiency of the Fur protein. On the basis of this consideration, the relative intensities of the peaks in the mass spectra, representing the relative proportion of gas phase ions, were also indicative of the relative proportion of the different species in solution.

Figure 4A shows reconstructed mass spectra of HpFur WT obtained at various times during alkylation. The spectrum obtained before the beginning of the reaction shows the presence of one species at 17680.8 Da, precisely corresponding to the mass of the peptidic chain calculated from the amino acid sequences (17681 Da). The absence of the dimeric form or of zinc atom can be explained by the denaturing conditions used to obtain the mass spectra with a good sensitivity. After alkylation for 1 min of the HpFur WT (Figure 4A), mainly two new peaks appear with 57 and 114 Da mass increases. They correspond to the addition of one and two alkylating groups to HpFur WT, thus indicating that two of the cysteines are more reactive. As the reaction continues (15−60 min), peaks corresponding to the addition of six alkylating groups steadily increase whereas peaks corresponding to Fur and [Fur + (2 × 57)] progressively decrease and have disappeared after alkylation for 30 min. After alkylation for 30 min, all six cysteines are alkylated. It is noteworthy that [Fur + (3 × 57)], [Fur + (4 × 57)], and [Fur + (5 × 57)] species almost did not appear. To determine if the four slow-reacting cysteines in HpFur WT were protected due to their zinc binding, we performed the alkylation in the presence of EDTA. It was supposed that this divalent cation scavenger could trap the Zn^{2+} ion and suppress the protection effect. The addition of a large excess of EDTA dramatically changed the alkylation kinetics obtained through the different mass spectra presented in Figure 4C.

In the presence of EDTA the peaks corresponding to [Fur + (3 × 57)] and [Fur + (4 × 57)] directly appear for HpFur WT after 1 min, and alkylation on the six cysteines is almost completed after only 15 min. It indicates that the four cysteines involved in zinc binding became accessible to the alkylating reagent after removal of the zinc by EDTA treatment. The same experiment, realized on one double mutant of HpFur "C78SC150S", will be discussed below (Figure 4B).

Identification of the Two Free Cysteines. To identify the two cysteines which were more reactive in the absence of EDTA,

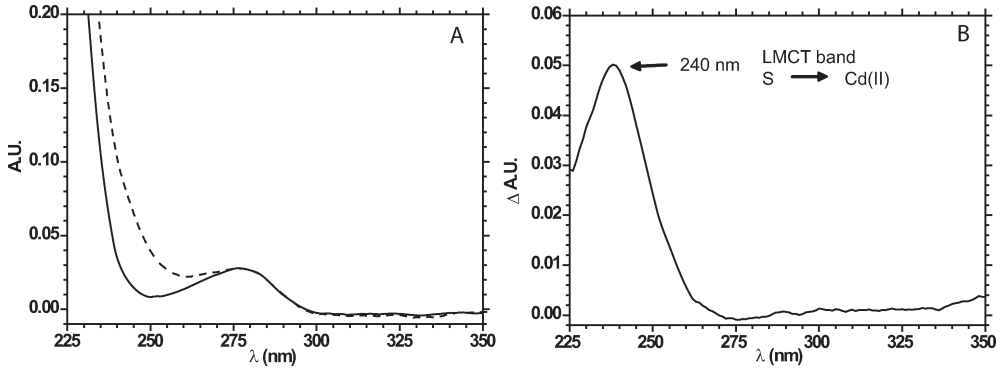


FIGURE 5: Cadmium binding by the dimeric HpFur C78SC150S double mutant. (A) Absorption spectra of apo-HpFur C78SC150S and Cd(II)-HpFur C78SC150S. (B) Difference spectra between 1 equiv of Cd(II) per monomer of HpFur C78SC150S and apo-HpFur C78SC150S. Titrations were performed in 20 mM HEPES, pH 7.4, and 500 mM NaCl at 20 °C. The same spectra were obtained for the WT protein.

specific proteolysis of the sample containing a mixture of species HpFur WT + (1 × 57) and HpFur WT + (2 × 57), obtained after alkylation for 1 min (Figure 4A), was cleaved with endoproteinase Lys-C. MALDI mass spectra of the crude mixture of the resulting peptides are presented in Supporting Information Figure S2. Different peptides obtained with Lys-C contain one or two cysteines. Their alkylation states have been analyzed and confirmed that Cys78 is one the first cysteines alkylated in HpFur, whereas Cys102, Cys105, and Cys142 are not alkylated in the mixture species HpFur WT + (1 × 57) and + HpFur WT (2 × 57).

The C78SC150S double mutant was constructed in order to confirm the mass data, suggesting the involvement of the four cysteines, 102, 105, 142, and 145, in the zinc binding site. It also contains one zinc/subunit (PAR and ICP-AES analysis) and behaves as the WT protein during the purification but is more stable (less oligomerization and precipitation). The circular dichroism spectra of WT and the C78SC150S double mutant are identical and slightly different of the EcFur spectrum recorded in the same conditions (Supporting Information Figure S3). It indicates a mixed α-helix and β-sheet secondary structure with a significant degree of α-helical structure, in agreement with the PaFur dimer structure (42% of α-helices), the BsPerR, and the EcFur. Moreover, this nuclease protection assay confirms that HpFur C78SC150S behaves exactly as the wild type (Figure 3).

The kinetics of alkylation of the C78SC150S double mutant is slower than for the HpFur WT with still a large amount of nonalkylated form after 60 min (Figure 4B). This variation is due to the absence of the two more reactive cysteines "Cys78 and Cys150". Moreover, we can observe a two by two behavior for the four cysteines. They are paired. The effect of EDTA is really drastic and almost all of the four cysteines are alkylated after 1 min of reaction (Figure 4D).

Upon Cd(II) addition to the apoprotein monomer (WT or C78SC150S double mutant) obtained after EDTA treatment in the presence of reductant, an absorption band of the cadmium-substituted protein presented in Figure 5 clearly shows an absorbance at 240 nm corresponding to a S → Cd(II) ligand to metal charge transfer band. We obtained an absorption coefficient value of 22610 $M^{-1}\cdot cm^{-1}$ at 240 nm for the C78SC150S double mutant and a slightly lower value 20180 $M^{-1}\cdot cm^{-1}$ for the WT protein. This is probably due to poorer stability of the WT monomer. Considering an average value of 6000 $M^{-1}\cdot cm^{-1}$ (*43*) for one thiolate bound to a Cd(II), this confirms the presence of the four thiolate-containing ligands bound to the cadmium ion.

DISCUSSION

The secondary structure prediction of HpFur suggests a similar folding than the other members of the Fur family such as EcFur and BsPerR. However, differences are clearly demonstrated between the structural zinc sites of these members of the Fur family: a ZnS_4 site in the BsPerR (*39*, *44*) or MtZur (*45*), a ZnS_2N/O_2 site in EcFur (*18*, *21*), a ZnS_3N/O_1 in EcZur (*46*), and a previously described ZnN_2O_2 site in PaFur (*16*) (now reassigned to the Fe(II) sensing site (*26*, *47*)). Table 1 summarizes the properties of Fur and Fur-like proteins for which information about the structural zinc site has been described.

The sequence alignments of those proteins show a conservation of two CxxC motifs between HpFur, BsFur, AbFur, BsPerR, and MtZur (FurB). Some of them have been shown to contain a ZnS_4 site (*39*, *44*, *45*), but some others only intrasubunit disulfide bridges (*48*). HpFur contains six cysteines which could be involved in metal binding or disulfide bridges. In this work, purification of native HpFur without any tag has been performed using a protocol which conducts to pure dimeric protein containing one zinc ion per subunit. This dimeric state is in agreement with former oligomerization studies (*10*) (Scheme 1).

This protein was able to be activated by Co(II) dication (which mimics easily oxidized Fe(II)) for specific DNA binding on Fur boxes contained in the *exbB* promoter. Several overlapping Fur boxes can be predicted in this promoter (data not shown) which correspond to the footprinting (*29*). However, we observed no binding to the contiguous NikR binding site, confirming the specificity of the interaction and the absence of polymerization on this side of the promoter. We did not observe any binding to this promoter in the absence of metal traces. The fact that apoHpFur was able to bind specifically the promoter of its own gene without metal activation (*10*) is still questionable from a structural point of view.

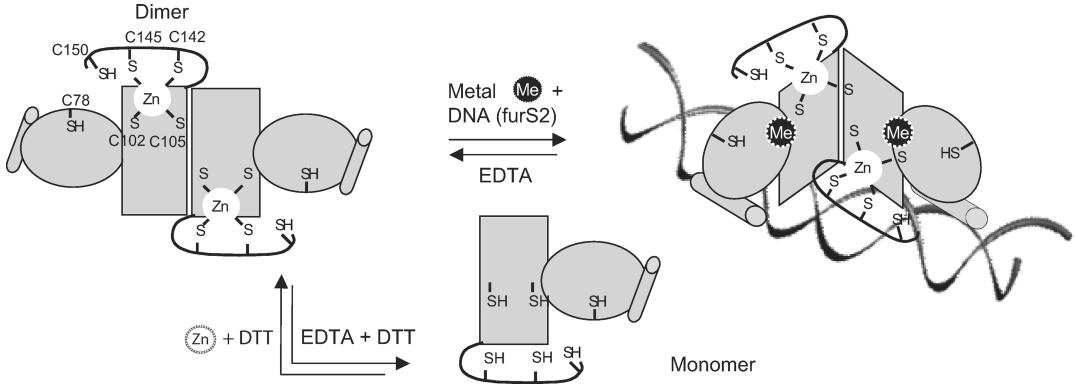
Using chemical modifications of the thiolate groups together with mutagenesis of two of the six cysteines and biochemical characterization of the oligomeric state of the protein, we demonstrate that HpFur contains a ZnS_4 structural zinc site essential to the dimerization comparable with the BsPerR structural zinc site. The strong chelating properties of the two CxxC motifs of this structural zinc site explain the higher stability of the HpFur dimer comparatively to the EcFur dimer in the presence of EDTA. The two other cysteines, C78 and C150, are shown not to be essential to the zinc binding, to the dimerization, and to the specific DNA binding. These two cysteines, highly reactive to the chemical modification, should be solvent exposed. One of them has been clearly identified as C78, and our data

Table 1: Properties of Fur and Fur-like Proteins for Which Information about the Structural Zinc Site Has Been Described[a]

EcFur	----------**C**xx**C**----------Cxxxx**C**---	ZnS_2N/O_2 structural Zn(II) essential to dimerization (*18, 21-23*)
PaFur	----------CxxT----------	no cysteine bound Zn(II) (*50*) (*16*)
VhFur	-----C----CxxC----------Cxxxx**C**---	(*24*)
ValFur	-----C----**C**xxC----------Cxxxx**C**---	(*51*)
VanFur	-----C----CxxC----------Cxxxx**C**---	ZnSx reduced thiol important for Zn and DNA binding (*52*)
EtFur	-----C----**C**xx**C**----------Cxxxx**C**---	(*25*)
BsFur	---C------CxxC----------CxxC-----	(*53, 54*)
AbFur	----------CxxC-------C--CxxC-----	no zinc, intrasubunit disulfide bridges, oligomerization (*48*)
HpFur	-----C----CxxC----------CxxC--C--	ZnS_4 essential to dimerization (**this work**)
BsPerR	----------**C**xx**C**----------**C**xx**C**-----	ZnS_4 essential to dimerization (*39, 44*)
SrFurS	----------**C**xxC----C--C--CxxC-----	ZnSx oxidation of the first CxxC motif induces Zn release (*55*)
MtFurB	--C-------CxxC----------CxxC--C--	ZnS_4 (MtFurB=Zur) (*45*)
EcZur	-C---C----CxxC----C-----CxxC--C-C	$ZnS_3(N/O)$ (*46*)

[a] Overlined cysteines in gray have been shown to be bound to a zinc ion either by X-ray crystallography or using EXAFS or mass analyses studies after chemical modification. C represents the cysteine residue in the protein sequences schematized by − − −. In bold are presented the cysteines which are essential to the metal or DNA binding activities. Underlined are presented the cysteines which are not essential to the activity as demonstrated by mutagenesis. Abbreviations: EcFur, *E. coli* Fur; PaFur, *P. aeruginosa* Fur; VhFur, *V. harveyi* Fur; ValFur, *Vibrio alginolyticus* Fur; VanFur, *Vibrio anguillarium* Fur; EtFur, *E. tarda* Fur; BsFur, *B. subtilis* Fur; AbFur, *Anabaena* PCC7119 Fur; HpFur, *H. pylori* Fur; BsPerR, *B. subtilis* PerR; SrFurS, *Streptomyces reticuli* FurS; MtFurB = Zur, *M. tuberculosis* Zur; EcZur, *E. coli* Zur.

Scheme 1: Schematic Summary Describing the Structural Metal-Binding Site, the Oligomeric State of HpFur, and Its Activation in Function of the Metal Binding



suggest that the other one is contained in the 131−150 peptide. The C142−C145 chelate-forming motif is probably bound to the zinc, suggesting that the last solvent-accessible cysteine is C150 (Scheme 1). The instability of the wild-type protein to air in the absence of reducing conditions and the observation of oxidation-dependent oligomerization may suggest a role of those two cysteines in a possible sensitivity of HpFur to oxidative stress conditions. We did not find any straightforward correlation, from our data and the literature, between the number and the positioning of the cysteines and Fur and Fur-like reactivity. However, the proteins containing a ZnS_4 site may have a more stable dimer in oxidative stress conditions. The presence of extra cysteines such as in HpFur may favor higher oligomeric states which may have some physiological roles such as oligomerization along DNA as it was demonstrated for EcFur (*49*) or inactivation by oligomerization.

However, the predicted secondary structure (Figure 1) was almost identical to the other members of the Fur family which structures were known and the identification of a $ZnCys_4$ structural zinc site suggest that we can predict the putative structure of HpFur using the homology model.

Homology models of HpFur from the crystallographically determined BsPerR have been built (Figure 6). The PerR structure was chosen for this model because it corresponds to the conformation of the inactive protein (no metal ions in the regulatory site), and PerR contains only four C in two CxxC motifs. It clearly shows that the two CxxC motifs would be in an ideal position to complex a Zn(II) in a tetrahedral geometry. Furthermore, this also suggests that the D98 and H99 amino, demonstrated as essential for activity (*10*), are part of the proposed regulatory site "S2" necessary for BsPerR and HpFur protein activities (not shown).

Iron homeostasis is particularly important in pathogenic bacteria such as *H. pylori*, which need to compete with the host for this essential cofactor. Iron homeostasis and oxidative stress defense are intimately linked and are both important for virulence. In *H. pylori*, HpFur which functions as a pleiotropic regulator (*12*) is one of the main links. Indeed, HpFur has been shown to control iron superoxide dismutase (sodB) expression by direct interaction of apoHpFur with its promoter for the repression of its expression (*30*). Furthermore, Fur-deficient mutant and cells under iron starvation condition showed a reduction in catalase activity and increased sensitivity to H_2O_2, suggesting a direct or indirect role for *fur* in regulating katA (catalase) expression in response to iron (*34*). Fur is also involved in iron-responsive regulation of

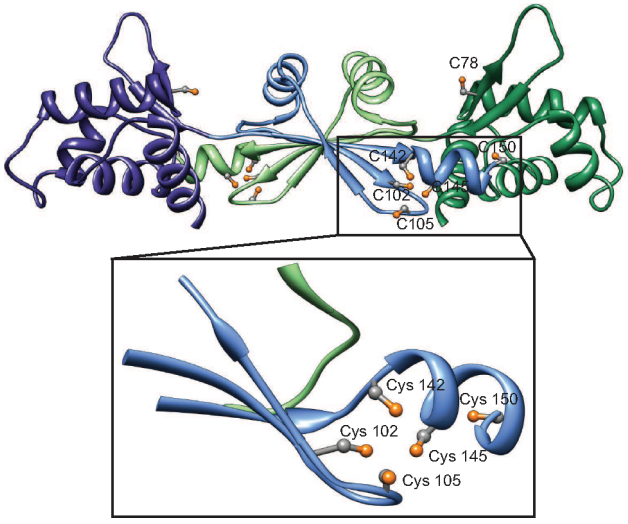


FIGURE 6: Structural model of native *H. pylori* Fur. A dimer of HpFur was modeled on the *B. subtilis* PerR structure (PDB code 2FE3) (*39*) using Modeler (*40*) after alignment with Clustal W (*41*). For more clarity only the six cysteine lateral chains per subunit are represented. The two different subunits are shown in green and blue colors. The two CxxC motifs containing the 102, 105, 142, 145 cysteines are well positioned for the zinc binding demonstrated in the present paper.

napA expression (Dps homologue; repression of NapA by iron starvation (*56*)).

The diverse antioxidant systems of *H. pylori* have been described (*57*) but a surprisingly low number of regulators, potentially involved in regulation of expression of antioxidant proteins. No homologues of the oxidative stress regulators present in other bacteria, including OxyR, SoxRS, RpoS, LexA, and PerR, have been found in the *H. pylori* genome (*58*). Fur is one of these regulators. CsrA, a posttranscriptional regulator, is another one. Interestingly, CsrA, which was necessary for full motility and survival of *H. pylori* under oxidative stress, was shown to regulate Fur and heat shock gene regulator HspR. This is one more example of the intricate regulations described between *H. pylori* regulators such as NikR, Fur, ArsR, and CsrA.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE of purified HpFur (Figure S1), MALDI-TOF mass spectra of peptides obtained by endoproteinase digestion by Lys-C (Figure S2), and circular dichroism spectra of HpFur (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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