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Structure and dynamics of *Helicobacter pylori* nickel-chaperone HypA: an integrated approach using NMR spectroscopy, functional assays and computational tools

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Abstract

Helicobacter pylori HypA (*HpHypA*) is a metallochaperone necessary for maturation of [Ni,Fe]-hydrogenase and urease, the enzymes required for colonization and survival of *H. pylori* in the gastric mucosa. *HpHypA* contains a structural Zn(II) site and a unique Ni(II) binding site at the N-terminus. X-ray absorption spectra suggested that the Zn(II) coordination depends on pH and on the presence of Ni(II). This study was performed to investigate the structural properties of *HpHypA* as a function of pH and Ni(II) binding, using NMR spectroscopy combined with DFT and molecular dynamics calculations. The solution structure of apo, Zn-*HpHypA*, containing Zn(II) but devoid of Ni(II), was determined using 2D, 3D and 4D NMR spectroscopy. The structure suggests that a Ni-binding and a Zn-binding domain, joined through a short linker, could undergo mutual reorientation. This flexibility has no physiological effect on acid viability or urease maturation in *H. pylori*. Atomistic molecular dynamics simulations suggest that Ni(II) binding is important for the conformational stability of the N-terminal helix. NMR chemical shift perturbation analysis indicates that no structural changes occur in the Zn-binding domain upon

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addition of Ni(II) in the pH 6.3–7.2 range. The structure of the Ni(II) binding site was probed using ^1H NMR spectroscopy experiments tailored to reveal hyperfine-shifted signals around the paramagnetic metal ion. On this basis, two possible models were derived using quantum-mechanical DFT calculations. The results provide a comprehensive picture of the Ni(II) mode to *HpHypA*, important to rationalize, at the molecular level, the functional interactions of this chaperone with its protein partners.

Keywords

Metallochaperones; Metal transport; Molecular dynamics; Nuclear magnetic resonance; Computational chemistry; Nickel

Introduction

Helicobacter pylori (*Hp*) is a bacterium that infects the stomach of a large proportion of the human population [1]. The bacterial induction of disease entails a chronic inflammation of the gastric mucosa that, in some cases, can progress to ulcers, gastric cancers and MALT lymphoma [2, 3]. For this reason, eradication of *Hp* infection is considered a significant step toward population-level cancer prevention and treatment [4, 5]. However, the available antibiotic-based treatment against *Hp* is becoming a blunted weapon, due to the increased occurrence of antimicrobial resistance. As such, the standard first-line intervention against *Hp* is already ineffective in 20% of the cases and, consequently, the World Health Organization (WHO) has included clarithromycin-resistant *Hp* on the list of global priorities for the development of new antibacterial molecules [6].

To survive in the acidic environment of the gastric mucosa, *Hp* relies on a unique adaptive mechanism that involves the rapid hydrolysis of urea catalyzed by the nickel-dependent enzyme urease, a reaction that maintains internal cellular pH and may increase the pH around the bacterium [7–12]. Indeed, urease-negative *Hp* strains cannot colonize the stomach mucosa of mice [13, 14]. Interestingly, the antibiotic treatment against *Hp* reaches significantly higher eradication rates if a nickel-free diet is maintained by infected patients, suggesting that the reduction of urease activity increases the bacterial susceptibility to antibiotics [15]. Therefore, inhibition of urease activity is an attractive target for the development of antibacterial strategies to overcome *Hp* gastric infection. The role of the dinuclear Ni(II)-containing active site of urease in the catalytic hydrolysis of urea is thought to lie in the need for the generation of the hydroxide ion acting as the nucleophile at a pH near neutrality, as well as the peculiar stereo-electronic properties of the open shell d^8 Ni(II) ion, required to place the two substrates (urea and hydroxide) in the correct orientation within the active site [12, 16].

The very specific requirement for Ni(II) in the activation of urease requires a very selective maturation process that encompasses the incorporation of two nickel ions into the active site of the enzyme, and is carried out by a multiprotein complex of four accessory proteins, named UreD, UreE, UreF and UreG [7, 8]. Other metal-dependent hydrolases, for example glycerophosphodiesterases, show a looser requirement for a specific element and instead depend on other more common and abundant microelements such as Fe and Zn to carry out

their catalytic reactions, explaining the lack of a specific and complex assembly mechanism [17]. For urease, UreF, UreD and UreG form an initial complex that pre-activates urease for nickel incorporation [8], while UreE serves as the metallochaperone that delivers Ni(II) to the target sites [12, 18–20]. This process is also facilitated by two additional metallochaperones, HypA and HypB [21], which are responsible, together with SlyD, for nickel incorporation into the active site of [Ni,Fe] hydrogenase [11, 22, 23].

The role of HypA in urease maturation is unique to *Hp* and is potentially mediated by the formation of a stable complex with dimeric UreE [24, 25]. The *Hp*HypA·*Hp*UreE₂ protein complex contains a unique high-affinity nickel-binding site that is not present in either component [24]. Deletion of *Hp*HypA [26–28] or mutations of residues in the conserved Met-His-Glu (MHE) N-terminal amino acid sequence (His2 or N-terminal modification) [21, 27] reduce the acid viability of *Hp* cells and lead to a loss of urease activity in *Hp* cell lysates, identifying the N-terminus as an important component of the nickel-binding site structure that is crucial for the formation of the high-affinity nickel-binding site in the *Hp*HypA·*Hp*UreE₂ complex [24, 27].

In addition to the nickel-binding site, HypA contains a unique structural zinc site that is associated with two conserved CXXC sequences that, in *Hp*HypA, are flanked by His residues. The presence of Zn in this site is essential to maintain the protein in solution in a stable form. X-ray absorption spectroscopic data suggested that the Zn(II) coordination sphere undergoes a structural rearrangement from Cys₄ ligation to Cys₂His₂ ligation in response to Ni(II)-binding and a pH decrease from 7.2 to 6.3 [29, 30]. However, while studies employing point mutations of the Cys and His residues in the Zn(II) site confirmed the importance of the zinc site and the Cys ligation for *Hp* acid survival and urease maturation, they failed to identify a specific role for the flanking His residues [23, 28].

Structures of HypA proteins have been obtained from NMR studies of an N-terminally modified construct of apo,Zn-HypA from *H. pylori* [31] (HypA*, PDB code 2KDX) and from X-ray diffraction studies of monomeric (PDB code 3A43) and dimeric (PDB code 3A44) apo,Zn-HypA from *Thermococcus kodakarensis* (*Tk*) [32], as well as of complexes of apo,Zn-*Tk*HypA formed with *Tk*HypB [33] or with the large subunit of *Tk* hydrogenase (HyhL, PDB codes 5YXY and 5YY0, respectively) [34]. These structures reveal the presence of an elongated HypA protein, where the zinc-binding site is well separated from the N-terminal MHE motif associated with the nickel-binding site, thus providing no evidence as to how the two metal sites might cooperate in nickel delivery to urease. The addition of Ni(II) to the modified apo,Zn-*Hp*HypA* led to the NMR characterization of a four-coordinate planar and diamagnetic Ni(II) site with ligands suggested to comprise the His2 imidazole and the amide N-donors from His2, Glu3 and Asp40 [31]. This conclusion contrasts with the characterization of the Ni(II)-binding site in the native apo,Zn-*Hp*HypA site by X-ray absorption spectroscopy (XAS) and magnetic studies as being six-coordinate and paramagnetic [27, 29]. This discrepancy has been traced to the presence, at the N-terminus of the modified apo,Zn-*Hp*HypA*, of a Gly-Ser N-terminal extension that induces the formation of an artifactual diamagnetic Ni(II) site [27]. The structures of three different complexes between *Tk*HypA and *Tk*HypB have also been reported [33], differing by (1) the presence of AMPPCP (a non-hydrolyzed ATP analogue bound at the interface between two

TkHypB monomers) and the absence of Ni(II) (PDB code 5AUP), (2) the presence of both AMP-PCP and Ni(II) (PDB code 5AUO), and (3) the presence of Ni(II) and ATP γ S, a non-hydrolyzable analogue of ATP (PDB code 5AUN). In both *TkHypA*·*TkHypB* complexes containing Ni(II), four-coordinate planar sites were characterized, with the N-terminal amine, the amide N atom of His2, and the side chains of His2 and His98 identified as nickel ligands [33]. However, such a structure is unlikely to exist in the case of *HpHypA*, because His98 is found in an extended loop in *TkHypA* that is not present in *HpHypA*. Thus, despite considerable protein structure information, the Ni(II) site structure in *HpHypA* is still lacking a detailed description. In addition, none of the protein structural studies to date have addressed acid shock conditions that are important to the rapid nickel addition to apo-urease [35], the latter comprising up to 10% of the total protein content in *H. pylori* [11].

Herein, we report a structural study of apo,Zn-*HpHypA* in solution carried out using 2D, 3D and 4D NMR techniques, in the absence and presence of Ni(II) and at pH 7.2 and pH 6.3, the latter value being an estimate of internal pH of *Hp* under acid shock conditions [36–38]. This choice was further supported by a more recent study that has quantified the cytosolic pH of *H. pylori* using a pH-sensing variant of green fluorescent protein called ratiometric pHluorin. The results indicate that the cytoplasmic pH of *H. pylori* cells exposed to a medium at pH 2, to mimic the conditions of the stomach, drops to a minimum value of 6.2 and recovers to neutrality, in the presence of Ni in the medium, within a matter of minutes [39]. The structure of apo,Zn-*HpHypA* at pH 7.2 was also calculated using a state-of-the-art NMR structure determination protocol. The NMR spectral analysis leads to a reassignment of some crucial NMR resonances that allowed us to exclude a communication between the nickel-binding and the zinc-binding domains. Hyperfine-shifted ^1H -NMR signals observed upon Ni(II) addition were used to characterize the ligands in the paramagnetic Ni site, and to develop possible models for the Ni(II) site structure based on quantum-mechanical calculations. These observations, together with atomistic molecular protein dynamics calculations starting from the structure of apo,Zn-*HpHypA* at pH 7.2 and proceeding for 100 ns, underscore the role of the Ni(II) binding in stabilizing the structure of the α -helix in the N-terminal region, likely facilitating the protein-protein interaction of *HpHypA* with partner proteins such as *HpUreE*, *HpHypB*, and the large subunit of hydrogenase.

Materials and methods

Protein production and purification

The expression and purification of [^{15}N]-*HpHypA* and [^{15}N , ^{13}C]-*HpHypA* were carried out using a previously described protocol [30], with some modifications. In particular, cells of *E. coli* BL21-pLysS (DE3) expressing *HpHypA* were cultured at 37 °C in 2 L of lysogeny broth (LB), collected by centrifugation when OD₆₀₀ reached 0.8, and then resuspended in 0.5 L of M9 minimal medium containing ($^{15}\text{NH}_4$)₂SO₄ with ^{12}C -glucose or with ^{13}C -glucose, as well as 5 μM ZnSO₄. After 10 min, protein expression was induced at 25 °C by adding isopropyl-thiogalacto-pyranoside (IPTG) to a final concentration of 0.5 mM. The culture was harvested 16 h after induction by centrifugation at 10,000g for 20 min at 4 °C and was resuspended in 30 mL of 20 mM Tris-HCl buffer at pH 7.2, containing 1 mM TCEP, 20 $\mu\text{g mL}^{-1}$ DNase, 1 mM MgCl₂ and 2 mM phenylmethylsulfonyl fluoride (PMSF)

as protease inhibitor. The cells were disrupted by two passages through a French pressure cell (SLM, Aminco) at 20,000 lb per square inch (PSI). The cell pellet was separated from the supernatant by centrifugation at 76,000g for 15 min at 4 °C and the soluble fraction was loaded onto a Q-Sepharose 16/10 column (GE-Healthcare) pre-equilibrated with 20 mM Tris-HCl buffer at pH 7.2, containing 1 mM tris(2-carboxyethyl) phosphine (TCEP). The column was washed using a flow rate of 2 mL min⁻¹ with the starting buffer until the baseline was stable. The protein was eluted from the column with a 220mL linear gradient of NaCl (0–0.6 M). Fractions containing *HpHypA* were combined, concentrated using 3-kDa Ultrafiltration units (Millipore) to a final volume of 2 mL, and loaded onto a Superdex 75 16/60 column equilibrated with 20 mM HEPES at pH 7.2 (or pH 6.3), containing 200 mM NaCl and 1 mM TCEP. Protein monomer concentration was estimated by the previously determined extinction coefficient at 280 nm ($\epsilon_{280} = 3250 \text{ M}^{-1} \text{ cm}^{-1}$) [27]. This protocol yielded ca. 15 mg of pure protein per liter of culture. Protein samples for NMR spectroscopy, constituted by ca. 1 mM [¹⁵N]- or [¹⁵N, ¹³C]-labeled apo,Zn-*HpHypA* in 20 mM HEPES at pH 7.2 (or pH 6.3), 200 mM NaCl, 1 mM TCEP, and 10% D₂O, were flash-frozen using liquid nitrogen, stored at - 80 °C, and thawed immediately prior to measurement. Throughout the paper, apo,Zn-*HpHypA* refers to the purified protein that contains 1 eq. of Zn and no other metal ions, as confirmed by inductively coupled plasma optical emission spectroscopy (ICP-OES) measurements carried out using a previously described methodology [40].

NMR spectroscopy data collection for protein structure determination

NMR spectra for backbone resonance assignment of apo,Zn-*HpHypA* at pH 7.2 were acquired using a Bruker AVANCE 900 MHz spectrometer, while spectra for side-chain resonance assignment and distance restraint extraction from NOESY spectra were recorded using a Bruker AVANCE 800 MHz spectrometer (Tables 1, 2–SI). In all cases, the spectrometers were equipped with 5 mm TCI-HCN z-gradient cryo-probes, and the temperature was calibrated at 298 K. Due to the high salt concentration, either shaped NMR tubes (Bruker BioSpin AG) or salt-tolerant susceptibility matched slot NMR tubes (Shigemi Inc.) were used at 900 or at 800 MHz, respectively, to improve the signal-to-noise ratio during NMR data collection. Proton chemical shifts were referenced to 2,2-dimethyl-2-silapen-tane-5-sulfonic acid sodium salt (DSS), while the ¹³C and ¹⁵N chemical shifts were referenced indirectly to DSS, using the ratios of the gyromagnetic constants.

The backbone and side chains H β and C β nuclei were assigned using 3D HNCO/ HN(CA)CO, HNCACB/ CBCA(CO)NH, and HBHANH/HBHA(CO)NH spectra. These spectra were processed using NMRpipe [41], and the sequence-specific assignment was carried out using the software CARA (<http://www.cara.nmr.ch>) [42]. Table 1-SI reports the details of all NMR spectra acquired for backbone signal assignment on apo,Zn-*HpHypA* at pH 7.2 and pH 6.3. The ¹H,¹⁵H HSQC spectra were also recorded in the presence of incremental amounts of NiSO₄ at both pH 7.2 and pH 6.3. The side-chain nuclei were assigned using 4D aliphatic HCCH-TOCSY, 2D (HB)CB(CGCD)HD and aromatic 4D HCCH-TOCSY spectra. The side-chain NH₂ groups of Asn and Gln residues were identified using NOEs connectivities with their respective H β or H γ protons. 4D [¹H,¹H]-NOESY experiments with mixing time of 120 ms were recorded in the ¹⁵N,¹⁵N-resolved, ¹³C_{ali}, ¹⁵N-

resolved, $^{13}\text{C}_{\text{ali}}$, $^{13}\text{C}_{\text{ali}}$ -reSolVed, $^{13}\text{C}_{\text{ali}}$, $^{13}\text{C}_{\text{aro}}$ -resolved and $^{13}\text{C}_{\text{aro}}$, $^{13}\text{C}_{\text{aro}}$ -resolved varieties. All 4D experiments utilized sparse sampling of indirectly sampled dimensions with on-grid sampling schedules randomly drawn from a truncated Gaussian distribution with the standard deviation set to half of the maximal evolution time. The spectra were processed using the Signal Separation Algorithm, implemented in the clean-er4d software [43]. The spectra were inspected and manually peak-picked using the UCSF Sparky software [44], and the side-chain resonances were manually identified using the sequence-specific backbone assignment.

Determination of the structure of apo,Zn-*HpHypA* at pH 7.2 using NMR spectroscopy

Input for the calculation of the apo,Zn-*HpHypA* structure at pH 7.2 was peak lists from five 4D [^1H , ^1H]-NOESY spectra recorded in the ^{15}N , ^{15}N -resolved (398 peaks), $^{13}\text{C}_{\text{ali}}$, ^{15}N -resolved (877 peaks), $^{13}\text{C}_{\text{ali}}$, $^{13}\text{C}_{\text{ali}}$ -resolved (3752 peaks), $^{13}\text{C}_{\text{ali}}$, $^{13}\text{C}_{\text{aro}}$ -resolved (256 peaks) and $^{13}\text{C}_{\text{aro}}$, $^{13}\text{C}_{\text{aro}}$ -resolved (53 peaks) modes, recorded at 800 MHz. In addition, 191 backbone dihedral angle restraints were obtained using an analysis of secondary chemical shifts carried out using TALOS+ [45]. Bond distances and angle constraints were used to bind the Zn(II) ion to the thiolate S γ of Cys74, Cys77, Cys91 and Cys94 (Zn-S distance of 2.35 Å as derived from previous XAS data [30] in a tetrahedral geometry with S-Zn-S angles of 109.5°. The latter was also enforced by distance restraints between S γ of coordinating cysteines (S-S distance of 4.0 Å).

The structure was determined with a new approach (named YARIA) that combines the iterative protocol for automated NOE assignment implemented in ARIA (Ambiguous Restraints for Iterative Assignment) software [46] with the YASARA (Yet Another Scientific Artificial Reality Application) molecular dynamics software [47]. In this particular case, the protocol comprised 11 iterations (it0-it10). Automatic iterative assignment of the NOE cross-peaks and conversion to distance restraints was carried out using the standard ARIA routines, using 4–4 constraint combination and network anchoring approach [48, 49] for the first four iterations (it0–it3). Adaptive violation tolerances [50] were used in all iterations. In each iteration, the restraints thus generated were used as input, together with dihedral angle restraints, for structure calculation using YASARA. Every iteration generated 30 conformers (except for the last one, where 100 structures were calculated) of which the 20 lowest energy conformers were selected for analysis.

In the first two iterations (it0–it1), each conformer was calculated using four steps. The first step involved folding from an extended conformation of the polypeptide using Monte Carlo sampling in internal coordinates. During the calculation, restraints were added sequentially, from short-to long-range, and the restraint violation energy was used to accept or reject the structure using a classic Monte Carlo criterion. Energy minimization of each conformer was thus carried out to remove high energy conformations. In the first iteration (it0), four initial conformers were generated, of which the minimized average was used as input for the subsequent refinement steps, while in the second iteration (it1) a single conformer was likewise generated and minimized. The latter structure was then used in the second step, which involved an in vacuo refinement consisting in several cycles of implosion/expansion, by reducing/restoring non-bonded interactions, followed by simulated annealing. Explicit

solvent refinement was then performed in the third step, using a water shell extending 8 Å around protein atoms, carrying out three cycles of heating to 298 K and annealing to 0 K. In these first three steps, NOE distance restraints were included with a flat bottom harmonic well potential where the scaling factor is automatically optimized (maximum value 50 kcal mol⁻¹). The fourth step consisted of ten cycles of simulated annealing using a bounds-free log-harmonic potential for distance restraints [49] where the force constant is automatically optimized according to the satisfaction of the restraints [50]. The YASARA force field [51] was employed in all YASARA calculations. It includes knowledge-based potentials for backbone and side-chain dihedral angles and Particle Mesh Ewald summation [52] for calculation of electrostatic interactions.

In the following iterations (it2–it10), the protocol was identical with two exceptions: the first step was omitted, and the initial conformer was selected among the best structures from the previous iteration; additionally, a full water box type simulation cell extending 5 Å around all protein atoms and neutralized with counter ions was used in iterations it7–it10.

The 20 lowest energy conformers obtained in the last iteration were selected to represent the NMR structures. The Protein Structure Validation Software (PSVS) suite (http://psvs-1_5-dev.nesg.org/) and the WHATIF software [53] were used to validate the structure ensembles, while the MOLMOL [54] and UCSF Chimera [55] programs were used to analyze and represent the structure. Statistical data on the structure determination are reported in Table 1. The ARIA and YASARA protocols used in this study are available here: <http://www.yasara.org/yaria.htm>.

Accession numbers

The chemical shifts of apo,Zn-*HpHypA* at pH 7.2 were deposited in the Biological Magnetic Resonance Bank (BMRB) under accession code no. 34257. The atomic coordinates for the bundle of 20 conformers used to represent the structure of apo,Zn-*HpHypA* at pH 7.2 were deposited in the Protein Data Bank (PDB) under the accession code 6G81.

Molecular dynamics simulations on apo,Zn-*HpHypA* at pH 7.2

The closest-to-average conformer of the NMR structure ensemble of apo,Zn-*HpHypA* was selected for further investigations through the use of molecular dynamics (MD) simulations. The most probable protonation states of titratable amino acids and the tautomeric state of histidine residues at pH 7.2 were assigned using the H++ 3.2 server [56–58]. The protein was embedded into a truncated octahedron water box using a 12-Å buffer zone of solvent. The resulting system consisted of ca. 40,000 atoms. The Amber ff99SB force fields [59] for the protein and the TIP3P model [60] for water were used, while known parameters were applied for the Zn(II) binding site [61]. The system was neutralized by adding Na⁺ ions using the *genion* program of the GROMACS 5.1.2 package [62–64]. Analogously, additional Na⁺ ions were placed in the water box to achieve the ionic strength used in the NMR experiments (200 mM). A total of 58 Na⁺ and 49 Cl⁻ ions were thus added to the water box. The system was energy minimized and then equilibrated at 300 K and 1 atm by performing 1 ns of gradual annealing using GROMACS 5.1.2. The geometry optimization was performed in four cycles. In the first two cycles, which comprised 800 steps of steepest descent

followed by 200 steps of conjugate gradient, the water molecules were relaxed while the position of the protein atoms was constrained using a harmonic potential with a force constant of $1000 \text{ J mol}^{-1} \text{ \AA}^{-2}$. In the third and in the fourth cycles, the procedure was repeated without applying any constraint. During this equilibration phase, positional constraints were applied on the protein atoms (force constant of $1000 \text{ J mol}^{-1} \text{ \AA}^{-2}$). Temperature and pressure were controlled using a Berendsen thermostat and barostat [65], respectively. An integration step of 1 fs was used, and the structures were sampled every 0.1 ps. Periodic boundary conditions were applied. The Particle Mesh Ewald method was used to calculate electrostatic interactions [66]. The cutoff values for the real part of the electrostatic interactions and for the van der Waals interactions were set to 10 Å. During the 100 ns-long production run, the temperature and pressure coupling was made using a Nose-Hoover thermostat [67, 68] and a Parrinello-Raman barostat [69, 70], respectively. Clustering analysis was performed using the *cluster* module of GROMACS, using the Gromos algorithm [71]. A 0.15 nm cutoff for the RMSD was used to include structures in the same cluster.

Site-directed mutagenesis of HypA glycine residues

Polymerase chain reaction (PCR) was used to introduce the desired glycine to alanine mutations in *HpHypA*. The pET22b (+) vector encoding the wild-type *hypA* sequence from *H. pylori* was used as the template DNA for single point mutations and PCR primers (Table 3-SI) were designed to incorporate those desired mutations. For each 50 µL volume of PCR mixture, 0.5 µM of each primer was used for 2 ng of template DNA. For inserting G34A/G104A double point mutation in HypA, primers for G34A HypA were used with the G104A HypA plasmid as the template. Similarly, for inserting G32A/G89A double point mutation in HypA, the G32A HypA plasmid was used as template with the G89A HypA primers. Successful PCR amplifications were determined using a 0.8% agarose gel and the amplicons were subsequently digested with DpnI for 1 h at 37 °C to remove any methylated template DNA. The digested PCR mixture was then transformed into Novablue competent cells with ampicillin (Fisher Scientific) selection. Single colonies were grown to saturation in 5 mL LB-miller broth supplemented with Ampicillin at 37 °C. Cells were pelleted at 13,000g for 5 min and the plasmids were isolated using the GeneJET plasmid miniprep kit (Thermo Fisher Scientific). Plasmid sequencing (Genewiz, Inc.) confirmed the successful mutations.

Construction of *H. pylori* mutant strains

Helicobacter pylori HypA mutant strains were constructed as previously described [27, 28]. All strains were derived from DSM1283, a strain of *H. pylori* G27 in which the *hypA* gene was replaced with a *kan-sacB* cassette [28]. The HypA G32A, G89A, and double G32A/G89A mutations were obtained by transforming DSM1283 with individual pET22b plasmids containing the encoded *hypA* mutation. To increase the likelihood of recombination events upstream of G34 and downstream of G104, the HypA G34A, G104A, and double G34A/G104A mutations were obtained by splicing by overlap extension (SOE) PCR [72]. Specifically, the mutated *hypA* genes were PCR amplified from the respective pET22b vectors using the PhypA_F and PhypA_R primers (Table 3-SI). Then, a 442 base pair upstream DNA fragment and a 451 base pair downstream DNA fragment were added to either end of each mutated *hypA* amplicon using SOE PCR. Wild-type *H. pylori* G27

(DSM1) genomic DNA was used as template to PCR amplify the upstream and downstream regions using the HypA2_Up_F/HypA2_Up_R and HypA2_Dn_F/HypA2_Dn_R primer pairs, respectively. The four “upstream-*hypA*-downstream” SOE constructs were then individually transformed into the interrupted *hypA* mutant (DSM1283). Double crossover events that replaced the *kan-sacB* cassette with the mutated *hypA* gene were selected on horse blood agar (HBA) plates containing 5% sucrose. Kanamycin sensitivity of the mutants was confirmed on kanamycin HBA plates. Integration of the mutant *hypA* gene was confirmed by PCR and sequencing using the primers HypA2_Up_F and HypA2_Dn_R (G32A, G89A, and G32A/G89A), or with HypA_Confirm_F and HypA_Confirm_R primers and HypA_seq_F and HypA_seq_R primers (G34A, G104A, G34A/G104A). The newly created *H. pylori hypA* strains were renamed DSM1476 (G34A), DSM1477 (G104A), DSM1478 (G34A/G104A), DSM1649 (G32A), DSM1650 (G89A), and DSM1651 (G32A/G89A) (see Table 3-SI for strains and primers).

***H. pylori* growth**

Helicobacter pylori strains were maintained at - 80 °C in brain heart infusion (BHI) broth (BD) supplemented with 20% (v/v) glycerol (CalBioChem) and 10% (v/v) fetal bovine serum (FBS, Gibco). Growth on plates was achieved on 4.4% (w/v) Columbia agar (Acumedia) supplemented with 5% (v/v) horse blood (HemoStat), 0.2% (w/v) β -cyclodextrin (Sigma), 10 $\mu\text{g mL}^{-1}$ vancomycin (Amresco), 2.5 U mL^{-1} polymyxin B sulfate (Sigma), and 8 $\mu\text{g mL}^{-1}$ amphotericin B (Amresco). Liquid growth was accomplished in Brucella broth (Acumedia) supplemented with 10% (v/v) FBS and 10 $\mu\text{g mL}^{-1}$ vancomycin, with shaking at 110 RPM. Growth on plates or in liquid was performed at 37 °C, under microaerobic conditions (10% CO₂, 5% O₂, and N₂ as balance) achieved using an Anoxomat (Advanced Instruments Inc). Where appropriate, selection was performed with 5% sucrose or 25 $\mu\text{g mL}^{-1}$ kanamycin (Gibco).

Acid viability assay

Acid viability assays were performed as described previously [23, 27, 28]. Briefly, overnight liquid cultures were used to inoculate 10-mL liquid cultures to an optical density at 600 nm (OD₆₀₀) of 0.05, which were then grown for 20 h to an OD₆₀₀ of approximately 0.9–1.0. One OD₆₀₀ unit of bacteria was pelleted at 2500–3000g, the supernatant removed, and the bacteria resuspended in 1 mL phosphate-buffered saline (PBS) at pH 6.0 or pH 2.3, with or without supplemented 5 mM urea. The resuspension solutions were made by adding urea from a fresh 100 mM stock (in PBS) as appropriate, and the pH was adjusted using 6 M HCl. Immediately after resuspension, a 100 μL aliquot of *H. pylori* was removed, serially diluted in Brucella broth, and 2 or 10 μL of each dilution was plated to determine the colony forming units (CFU) at T_0 for each condition. The remaining *H. pylori* were incubated at 37 °C for 1 h with shaking. After 1 h, 100 μL was removed, serially diluted, and plated to determine the CFU at T_{60} for each condition. Plates were incubated for 4–5 days to allow for visualization of individual colonies. Percent survival was calculated as $T_{60}/T_0 \times 100$ for each condition and strain. Three biological replicates were performed.

Urease assays

Helicobacter pylori were grown as previously described [23, 27, 28]. For each strain, an overnight liquid culture of *H. pylori* was used to inoculate a 10-mL liquid culture to an OD₆₀₀ of 0.05. Cultures were allowed to grow for approximately 20 h, and 1 OD₆₀₀ unit of bacteria (approximately 10⁸ cells) was pelleted and frozen at - 80 °C prior to lysis.

Prior to urease assays, the frozen cells were thawed, resuspended in 750 µL of ice-cold lysis buffer (50 mM HEPES, pH 7.0, 1 mM phenylmethanesulfonic acid (PMSF) and 1× protease cocktail inhibitor from Sigma-Aldrich) and then lysed by pulsed sonication (500 Hz) at 40% amplitude for 12 s (2 s each pulse) on ice. The lysed cells were centrifuged at 14,000g for 15 min at 4 °C to remove insoluble particles from the soluble whole cell extract. Total protein concentration in the soluble whole cell extract was determined using the Bradford assay with the Coomassie Protein Assay kit (Thermo Scientific).

A modified phenol-hypochlorite method was used to assay the ammonia released from the soluble whole cell extract of *H. pylori* strains in the presence of urea. Briefly, 5 µL of the soluble whole cell extract of each strain was added to 245 µL of urease reaction buffer (50 mM HEPES, 25 mM urea, pH 7.0) and the mixture was incubated for 20 min at 37 °C to allow production of ammonia in solution. The reaction was quenched with sequential addition of 375 µL of Quenching Buffer A (100 mM phenol, 167.8 µM sodium nitroprusside) and 375 µL of Quenching Buffer B (125 mM phenol, 0.044% NaClO). The assay samples were quickly vortexed with each buffer addition and then incubated at 37 °C for 30 min to allow for color development (conversion of ammonia to indophenol). The absorbance of each assay sample was measured at 625 nm. The ammonia released from each assay mixture was quantified using a standard curve prepared using known amounts of ammonium chloride solution (0.2–500 nmol) in place of whole cell extracts. The urease activity of *ureB* strain was treated as background and subtracted from the urease activity of other strains. Urease activity of mutant *hypA* strains was normalized to the *hypA*-restorant strain (*hypA-R*, DSM1295) whose urease activity was treated as 100%. All assays were conducted in triplicate for two independently grown cultures.

¹H NMR spectroscopy of Ni,Zn-*HpHypA*

The residue-specific chemical shift perturbations (CSP) for the ¹H,¹⁵N-HSQC spectra were calculated using the relationship $CSP(NH) = \sqrt{|\Delta H| + |\Delta N|/7}$ (or $CSP(NH) = \sqrt{|\Delta H| + |\Delta N|/5}$ for glycines), for glycines), where | H| and | N| are the absolute values of the chemical shift differences, induced by pH change or by Ni(II) binding, of the amide proton and nitrogen resonances, respectively [73]. The values of CSP were also calculated for the Cα and Cβ backbone nuclei using the analogous relationship $CSP(C\alpha, C\beta) = \sqrt{|\Delta C\alpha| + |\Delta C\beta|}$ and the chemical shifts derived from the HNCACB/CBCA(CO)NH pairs of spectra in all four conditions examined. Figure 5 shows the results of this analysis.

¹H NMR experiments tailored to the identification of hyperfine shifted and fast relaxing signals [74] were performed on an AVANCE 400 Bruker NMR spectrometer, equipped with a 5-mm ¹H selective probe and operating at 400.13 MHz ¹H Larmor Frequency. Spectra

were collected with the superWEFT pulse sequence, using 52 ms, 20 ms and 62 ms as acquisition, recovery and inter-pulse delays, respectively. The spectral window was 156 kHz (390 ppm). The number of acquired scans ranged from 4 to 32 K, and the experiment time was typically 10–60 min. Prior to Fourier Transform, FIDs were multiplied by a cosine square weighting function followed by a 20 Hz Lorentzian line broadening. Phase and baseline correction were performed manually. Similar 1D experiments were performed also on AVANCE 700 and AVANCE 950 spectrometers operating at 700.13 MHz and 950.13 MHz ^1H Larmor frequency, respectively, in order to analyze the field dependence of the signal linewidths, using triple resonance inverse detection cryo-probes equipped with pulse field gradients along z . Values of T_1 for the hyperfine shifted signals were obtained by performing 15 inversion-recovery experiments, with an overall recycle delay of 72 ms and recovery delays of 0.1, 0.2, 0.5, 0.8, 1.2, 1.6, 2.0, 2.5, 3.5, 5.0, 7.0, 10, 15, 20, and 65 ms. Each 1D experiment was collected with 32 K scans, and spectra were processed as described above. 1D NOE difference spectra were obtained following a previously reported approach [75, 76]. 1D NOE difference experiments typically consisted of 0.8–2 million scans, each lasting typically 24–72 h.

Quantum mechanical calculations of the Ni(II)-binding site in Ni,Zn-*HpHypA*

The initial models for the structure of the Ni(II) coordination sphere in *Ni,Zn-HpHypA* were built using the Spartan '16 Parallel Suite v. 2.0.9. Density functional theory (DFT) computations were carried out using the program ORCA 4.0.1 [77] and the Becke three-parameter hybrid functional combined with Lee-Yang-Parr correlation functional (B3LYP/G) [78, 79] as defined in the Gaussian software [80]. All atoms except nickel were described by the Pople-style 6–311(p,d) basis set [81]. The Ni(II) ion was described with the Los Alamos effective core potentials (LANL2DZ ECP) [82]. Frequency computations were executed to determine the nature of the critical points.

Results

NMR structure of apo,Zn-*HpHypA* at pH 7.2

The 2D ^1H , ^{15}N HSQC spectrum of a uniformly ^{15}N -labeled sample of apo,Zn-*HpHypA* at pH 7.2 is shown in Fig. 1a. The large spectral dispersion of proton signals, as well as the consistency between the number of cross-peaks and that of amino acid residues in the protein, unambiguously indicates that the protein is homogeneous, pure, and with a defined fold in solution. The assignment of these signals, together with backbone $\text{C}\alpha$, $\text{C}\beta$, $\text{C}(\text{O})$, $\text{H}\alpha$ and $\text{H}\beta$ nuclei, was obtained using a combination of $\text{HNCO}/\text{HN}(\text{CA})\text{CO}$, $\text{HNCACB}/\text{CBCA}(\text{CO})\text{NH}$ and $\text{HBHANH}/\text{HBHA}(\text{CO})\text{NH}$ triple resonance spectra at 900 MHz on uniformly ^{15}N , ^{13}C -labeled samples of apo,Zn-*HpHypA* at pH 7.2. Following the identification of the backbone resonances, the assignment of the side-chains nuclei was obtained using a combination of 2D and state-of-the-art 4D NMR experiments at 800 MHz. The structure was determined with a new approach (named YARIA) that combines the iterative protocol for automated NOE assignment implemented in ARIA software [46] with the YASARA molecular dynamics software [47].

The ribbon diagram for the closest-to-average structure belonging to the ensemble of the 20 best NMR solution conformations of apo,Zn-*HpHypA* at pH 7.2 is shown in Fig. 2a. The large number of NOE restraints per residue resulted in a well-defined structure with very low ensemble averages of backbone and heavy atoms, with RMSD values of 0.78 ± 0.18 Å and 1.02 ± 0.16 Å, respectively (Table 1). No residues are found with backbone dihedral angles in the disfavored regions of the Ramachandran plot, while all quality scores are indicative of a high-quality structure (Table 1). The structure reveals the presence of two domains: residues 71–103 appear to constitute a domain containing the Zn(II) ion (Zn-binding domain), and residues 1–68 and 107–117 constitute the Ni-binding domain, while residues 104–106 and 69–70 make up a linker between the two domains. The RMSD values for the backbone and heavy atoms within the two individual domains are significantly smaller than the overall RMSD (Table 1), indicative of two well-ordered domains with variable relative orientation. This is evident in Fig. 2b, obtained by superimposing the Ni-domains of the 20 best structures, and highlighting the apparent conformational space occupied by the Zn-domain through the linker. The sausage diagram in Fig. 2c shows that the larger conformational variability is localized at the N- and C-termini and in the loops, as expected. The topological diagram of the structure (Fig. 2d) reveals that the Ni-domain is constituted by α -helix 1 (residues 3–20) and α -helix 2 (residues 41–52) flanking a three-stranded long parallel/antiparallel β -sheet made of β -strand 1 (residues 23–34), β -strand 2 (residues 62–68) and β -strand 6 (residues 107–116). On the other hand, the Zn-domain is made of β -strand 3 (residues 71–74), β -strand 4 (residues 77–81) and β -strand 5 (residues 99–103) that form a three-stranded short antiparallel β -sheet, in addition to a short loop connecting β -strands 3 and 4 and a long loop connecting β -strands 4 and 5, hosting the two pairs of nearby cysteine residues 74/77, and 91/94 that bind the Zn(II) ion through their thiolate groups.

Atomistic molecular dynamics calculations on apo,Zn-*HpHypA*

To gain a deeper understanding of the dynamic behavior of apo,Zn-*HpHypA*, a 100 ns-long MD simulation in explicit solvent was carried out using an atomistic force field and starting from the closest-to-average conformer of the NMR structure ensemble for apo,Zn-*HpHypA* at pH 7.2. The RMSD of the Ca atoms of both domains appears to be converged (Figure 3–SI) at values close to 0.2 nm after few ns of simulation time, while the Ca RMSD for the whole protein stabilizes at values close to 0.4 nm. The larger RMSD values observed for the protein backbone with respect to the isolated domains are due to the mutual movement of the two metal-binding domains and are not ascribed to large changes in the protein fold. Indeed, the secondary structure content is generally maintained in the course of the simulation (see Figure 4A–SI). However, the first 5–6 residues at the N-terminus, partially containing the α -helix and involved in Ni(II) binding, show a considerable degree of instability and a continuous series of folding-unfolding events occurring in the ns time scale (see Figure 4B–SI). This is consistent with the large fluctuations of the protein's Ca observed in the N-terminal region (Figure 5–SI), while other relevant fluctuations are observed in the loops of the Zn-binding domain (residues 75–80, 84–89 and 92–98) and, to a minor extent, at the C-terminus. A cluster analysis revealed that ca. 42% of the backbone conformations observed along the simulation can be ascribed to three clusters of structures accounting for 35, 16 and 15% of the simulation time (Figure 6–SI). The rest of the conformers can be grouped in an additional 47 clusters, accounting for a remaining 40% of

the simulation time, while 18% of the conformers cannot be assigned to any cluster. The most representative structure of the highest populated cluster of structures, present in the first 80 ns of simulation, has an interdomain angle, defined as the angle (β) formed by Glu117 C α , Glu106 C α and the Zn(II) ion, of 94°. In the second cluster, present in the 5–40 ns interval, the most representative structure has β =89°; while in the third most populated cluster, observed in the last 30 ns of simulation, β = 106°. The β angle in the remaining clusters range from 77° to 134°, with the interdomain angle changing in the tens of nanoseconds time scale. The current simulation thus explores and even extends the conformational space observed in the NMR structure ensemble (β from 120° to 142°), while additional conformations featuring larger β values obtained in longer time scales cannot be excluded.

The SPECTRUS method [83], which analyzes the fluctuations of all pairwise amino acid distances, revealed that apo,Zn-*HpHypA* can be divided in four quasi-rigid domains (QRDs, Fig. 3). These domains correspond to regions whose internal geometry is largely maintained while the relative interdomain position and orientation changes significantly. In other words, the large-scale motions of apo,Zn-*HpHypA* can be ascribed to the relative movement of these four QRDs, which are approximately rigid, although not static: QRD1 corresponds approximately to the Ni-binding region (residues 1–10 and 39–54), QRD2 comprises the largest part of the remaining residues in the nickel-binding domain (11–29, 55–63, and 112–117), QRD3 includes the residues in the region between the nickel- and the zinc-binding domains (30–38, 64–71, 82, and 103–111), and QRD4 (72–81 and 83–102) corresponds to the zinc-binding domain with the exception of Lys82. The latter remains H-bonded with Glu71 for ca. 50% of the simulation time and is thus comprised in QRD3. Motion correlations between various subparts of apo,Zn-*HpHypA* protein can be identified by a calculation of the covariance matrix of the amino acids displacements. Visual inspection of the corresponding map (Figure 7–SI) indicates that the motion of the Zn-domain (residues 66–103) and the other parts of the protein are anticorrelated, i.e., they move in a scissor-like movement.

Evaluation of physiological ramifications of *HpHypA* protein dynamics

The *HpHypA* protein contains four glycine residues that might confer flexibility to the linker region (Gly32, Gly34, and Gly104) and the Zn-binding domain (Gly89). Two of these Gly residues are conserved (Gly34 and Gly104), while two are non-conserved (Gly32 and Gly89) among HypA homologs. To assess the physiological importance of the flexibility of the linker region and Zn-binding site, mutant strains of *H. pylori* expressing HypA variants with altered flexibility afforded by Gly-to-Ala substitutions were created for each Gly residue in the HypA protein, as well as for pairs of conserved (G34A/G104A) and non-conserved (G32A/G89A) Gly residues, and the mutant strains were assessed for acid viability and urease activity. The results of acid viability assays of the mutant strains of *H. pylori* are summarized in Fig. 4. All strains survived similarly at pH 6.0, independent of the presence of exogenous urea (Fig. 4a, b). At pH 2.3 without urea, all strains were highly impaired for survival (Fig. 4c). At pH 2.3 in the presence of urea (Fig. 4d) the wild-type strain (WT) survived, due to its utilization of urea to neutralize the low pH. Under the same conditions, the survival of the *ureB* mutant, which does not have a functional urease, was

significantly impaired. The *hypA* mutant, which carries a *kan-sacB* cassette in the *hypA* coding region, was acid sensitive, as observed previously [27, 28]. The *hypA* restorant, as well as all of the Gly-to-Ala variants studied, did not show differences in survival from the WT strain under these conditions. Thus, it can be concluded that the Gly residues, and any flexibility that might be conferred by them, do not play a role in acid survival. Similarly, the results of *in vitro* urease assays on cell lysates from mutant strains of *H. pylori* expressing the Gly-to-Ala variants showed that urease activity in the variants was generally indistinguishable from the restorant strain (see Figure 8–SI).

Analysis of chemical shift perturbations upon pH change and Ni(II) binding

With the goal of assessing the structural changes induced by pH and by Ni(II) binding to apo,Zn-*HpHypA*, the NMR chemical shifts of backbone nuclei were investigated. The assignment of the amide NH signals in the ^1H , ^{15}N HSQC spectra of uniformly ^{15}N , ^{13}C -labeled samples of apo,Zn-*HpHypA* at pH 6.3 and 7.2, in the absence and presence of one equivalent of Ni(II) was obtained using a combination of HNCACB and CBCA(CO)NH triple resonance spectra at 800 MHz, following the assignment of the same signals for apo,Zn-*HpHypA* at pH 7.2. This procedure avoided assignment errors that might result from the use of a simple closest-neighbor criterion.

In all four cases, the N-terminal Met1 $-\text{NH}_3^+$ group was not observed because of rapid exchange with water that shifts its signal under the large water envelope, while Pro83 is devoid of the backbone NH moiety. The amide signal of His2 is also not observed in the spectra of apo,Zn-*HpHypA* at both pH values, likely because it is undergoing chemical exchange phenomena on an intermediate time scale. However, the signals of C α and C β of His2 could be detected using the NH signal for Glu3 in the ^1H , ^{15}N HSQC spectrum and the CBCA(CO)NH spectrum of apo,Zn-*HpHypA*, revealing no significant changes of chemical shift (0.7 ppm for C α and 1.1 ppm for C β) induced by pH. The intensity of the NH and C signals of residues in the range 53–59 is significantly smaller than expected for all four investigated conditions, suggesting the presence of dynamic conformational exchange in this region on the NMR time scale. The ^{13}C chemical shift of Cys C β nuclei depends on the redox state of the terminal S atom, being < 32 ppm for the reduced thiol state and > 35 ppm for the oxidized disulfide state [84]. According to this criterion, both Cys14 and Cys58, in the Ni-binding domain, appear to be in the reduced thiol form in all four states investigated (C β consistently at ca. 26 ppm), with no indication of the presence of a disulfide bond even though they are in close proximity in the structure. In the case of Cys74, Cys77, Cys91 and Cys94, found in the Zn-binding domain, the value is higher than expected for a thiol moiety (C β at ca. 32 ppm), which can be explained by the presence of Zn(II) bound to those thiolate groups.

The small values of the chemical shift perturbations (CSP) related to NH pairs [CSP(NH)] and C α ,C β pairs [CSP(C α ,C β)] calculated for apo,Zn-*HpHypA* as a function of pH, shown in Fig. 5a, b, respectively, indicate that no major rearrangement of the protein structure occurs in this pH range. In addition to the N- and C-terminal regions affected by pH because of the presence of weak acid and weak base $-\text{NH}_3^+$ and $-\text{COO}^-$ functionalities, relatively larger changes are observed in the vicinity of His2, His17, His24, His79 and His95. This is

not surprising, considering that His residues have their expected pK_a in this pH range. The values of CSP(NH) and CSP(C α ,C β) upon pH change (Fig. 5a, b vs. g, h) are noticeably smaller, on average, than the effect of Ni(II) binding (Fig. 5c, d vs. e, f), indicating that the protein structure is more sensitive to metalation rather than to pH.

Considering the proposed hypothesis of a communication between the Ni-binding and the Zn-binding domain [29, 30], special attention was dedicated to the values of CSP(NH) and CSP(C α ,C β) for the cysteine residues bound to Zn(II), and their flanking histidines, as a function of pH and Ni(II) binding. Figure 6 shows a comparison of the ^1H , ^{15}N HSQC signals for all His and Cys residues in the Zn-binding domain, while Table 2 reports their backbone N, H, C α and C β chemical shifts. The essential invariance of chemical shifts under the explored conditions indicates that no change in the Zn(II) ion coordination environment occurs in solution upon Ni(II) binding in the 6.3–7.2 pH range.

Ni(II) addition to apo,Zn-*HpHypA* at both pH 7.2 and Ph 6.3 causes large CSP(NH) and CSP(C α ,C β) in the region close to the N-terminus (Fig. 5c–f), and most notably the disappearance of twelve signals in the ^1H , ^{15}N HSQC spectrum, corresponding to residues Glu3, Val7, Ser8, Ala38, Met39, Asp40, Lys41, Ser42, Leu43, Phe44, Val45, and Ala47 (see Fig. 1b). These residues, when mapped on the NMR structure of apo,Zn-*HpHypA* (Fig. 7), identify a region in the Ni-binding domain where the paramagnetism of Ni(II) causes the broadening of the NH signals beyond detection. The Ni(II)-induced chemical shift changes for ^1H nuclei are significantly smaller than those for ^{15}N nuclei, indicating that there are no substantial contributions to chemical shifts arising from the pseudo-contact term; indeed, the latter does not depend on the gyromagnetic ratio, in contrast with the contact shift or the diamagnetic shift that arise from conformational changes [85]. This is an indication of a small magnetic susceptibility anisotropy around the $S = 1$ Ni(II) ion. The nature of the Ni(II) binding site has thus been investigated using ^1H NMR spectroscopic measurements tailored to highlight the hyperfine-shifted proton resonances arising from the paramagnetic center.

^1H NMR spectroscopy of paramagnetic Ni,Zn-*HpHypA*

The 400 MHz ^1H NMR spectrum of Ni,Zn-*HpHypA* is reported in Fig. 8 as a function of pH. No differences, even at small extents, are observed when pH is increased from 6.3 to 7.2, indicating that no structural/electronic/conformational changes occur in the Ni(II) first coordination sphere over this pH range, consistent with the lack of a structural change observed by prior pH-dependent XAS studies of the Ni,Zn-*HpHypA* Ni site [29, 30]. Eight signals, labeled A–H, are observed in the range from + 80 to - 40 ppm and outside the bulk diamagnetic region. The chemical shifts, longitudinal relaxation times T_1 , and line widths of these signals (Table 3), as well as the Curie-type temperature and field dependence of the chemical shifts (Figures 1, 2-SI) are consistent with the presence of a single paramagnetic Ni(II) center with $S = 1$ in octahedral coordination [86], as also deduced from XAS and Evans NMR magnetic measurements [27]. This is also consistent with the small magnetic anisotropy as derived by the comparison of the ^1H , ^{15}N HSQC spectra of apo,Zn- and Ni,Zn-*HpHypA*, mentioned above. The large values of the observed chemical shifts and the presence of signals both upfield and downfield with respect to the diamagnetic region of the spectrum suggest that pseudo-contact paramagnetic contributions are operative only in the

first coordination sphere of the Ni(II) chromophore in Ni,Zn-*HpHypA* [86]. The number of signals affected by hyperfine shift in the region from + 20 to - 10 ppm is considered an indication for the coordination geometry around a Ni(II) site in proteins: tetrahedral geometry determines a large magnetic anisotropy and thus the presence of many relatively sharp signals is affected by small pseudo-contact shifts [87]; octahedral coordination induces broad lines and small pseudo-contact shifts [88], while penta-coordinated Ni(II) is expected to produce an intermediate number of signals [89].

Only signal A disappears when samples are dissolved in D₂O solutions, indicating that it arises from an exchangeable HN proton. Chemical shift and linewidth of signal A are consistent with its assignment as an HN from the imidazole ring of an Ni(II)-bound histidine [86, 87, 89], indicating unambiguously that only one His is bound to this metal center. Considering all evidences that the N-terminus of *HpHypA* is involved in Ni(II) binding, and that only His2 is found in this region, the only possible assignment for signal A is that of a HNδ1 or HNE2 of the imidazole ring of His2.

1D NOE experiments, reported in Fig. 9, provide proton-proton dipolar couplings involving residues of the first coordination sphere of Ni(II). The selective saturation of signal A (Fig. 9a) shows a very weak NOE with signal D, and two other NOEs in the diamagnetic regions with signals at 2.24 ppm (1.3%) and 1.69 ppm (1.4%), respectively. An analysis of the spectrum with several different weighing functions confirmed the presence of a NOE connecting signals A and D, supporting the conclusion that they are coupled through dipolar interaction even though, considering the signal-to-noise ratio for the NOE experiment, a reliable quantitative estimation could not be performed (NOE < 0.8%). This supports the assignment of D as the vicinal partner of the exchangeable signal A of the imidazole ring of His2, namely either Cε1H or Cδ2H. This conclusion is supported also by the observation of an NOE signal observed at 1.69 ppm (1.3%) by saturating signal D (Fig. 9c), also observed upon saturation of signal A. The finding that signals A and D experiences a common NOE, together with the direct NOE from A to D, is strong evidence of their spatial proximity. The detection of a direct NOE from D to A (Fig. 9c) is prevented by the longitudinal relaxation rate R_1 of signal A. Indeed, the steady-state NOE on a signal I observed upon selective saturation of a signal J is given by

$$\eta_{I(J)} \propto \frac{\tau_m}{R_{1I}} r_{IJ}^{-6}, \quad (1)$$

where τ_m is the molecular tumbling correlation time, r_{IJ} is the distance between protons I and J , and R_{1I} is the longitudinal relaxation rate of signal I that gives NOE upon saturation of signal J . Therefore, steady-state 1D NOEs between two signals are not symmetrical but are scaled according to the R_1 values (Table 3). The NOE observed on signal A upon selective saturation of signal D is, therefore, expected to be about a factor of two smaller than the NOE observed for D upon saturation of A (Fig. 9a). The latter is already very close to the detection limit and, therefore, the reciprocal direct NOE from D to A in Fig. 9c is expected to fall beyond the detectability threshold of the experiment. This finding leads to the establishment of the binding mode of His2 to Ni(II): the relaxation rates of signals A and

D are of the same order of magnitude, indicating that they are both meta-like protons with respect to the imidazole-bound Ni(II), leading to the only possible assignment for signal A to His2 HNE2 and signal D to His2 HC82 protons. This reveals unequivocally that His2 is bound to Ni(II) through N81.

The selective saturation of signal C, shown in Fig. 9b, gives rise to an NOE with signal H (2.1%), a weak NOE with signal E (0.4%) and another NOE in the diamagnetic region at 1.94 ppm (0.7%). Considering a τ_m value of 7.8 ns (estimated using the empirical relationship τ_m (ns) $\sim$ 0.6 kDa [90]) and using the R_1 values measured for E and H (Table 3), we obtain from Eq. (1) a proton-proton distance of 1.8 ± 0.2 Å for protons corresponding to signals C and H, and 2.9 ± 0.3 Å for protons corresponding to signals C and E. These distances are only consistent with the assignment of signals C and H to a geminal βCH_2 or γCH_2 proton pair, and of signal E as αCH or a βCH_2 from the same residue. This is confirmed by the selective saturation of signal H (Fig. 9d), which shows again NOEs with E (1.1%) and with a signal at 1.94 ppm (1.0%), already observed upon saturation of signal C. Like the previously described situation, the predicted NOE from H to C is too small to be detectable. These data show that the signals C, E and H belong to the same spin system because of their relative spatial proximity. The analysis of their relaxation rates suggests metal-to-proton distances of 4.5–5.5 Å. Considering the involvement of Met1, His2 and Glu3, as well as Asp40, as likely ligands for Ni(II), the possible candidates for these signals are the side-chain protons of one of these four residues.

The absence of NOEs involving signals B, F and G prevents their assignment based on dipolar connectivities. Signal B features relaxation rates ca. one order of magnitude larger than the two meta-like protons. Under the approximation that the relaxation rates are dominated by the dipolar term ($R_{1,2} \propto r_{M-H}^{-6}$) and that unpaired spin density is metal-centered, the Ni-to-proton distances for signals B, F and G are estimated to be 3.7–4.4 Å, 5.2–5.9 Å and 4.7–5.3 Å, respectively. A structural model for the Ni(II) site is required to consider possible assignments for these signals.

DFT-based model of the Ni(II) site in Ni,Zn-HpHypA

The results of the analysis of the ^1H NMR spectra indicate that the Ni(II) binding site involves the N-terminus, the first three conserved triad of residues Met1-His2-Glu3, and the region surrounding Asp40. On this basis, and on the basis of previous XAS data on Ni,Zn-HpHypA [29, 30], two tentative structural models of the Ni(II) binding site were built by considering the known metal-binding atoms: Met1(N), His2(N), His2(N81), Glu3(N), Glu3(OE1), and Asp40(O81) (Fig. 10). Two similar models were built that feature the backbone N atoms of Met1, His2 and Glu3 in meridional configuration and the His2(N81) atom in an axial position but differ for the coordination geometry of Glu3(OE1) and Asp40(O81) with respect to His2(N81): in model **1**, Glu3(OE1) and Asp40(O81) are in trans- and cis-positions with respect to His2(N81), while in model **2** the positions of Glu3(OE1) and Asp40(O81) are swapped. Models **1** and **2** were optimized in the gas phase and the resulting structures are reported in Fig. 10, while selected structural parameters are reported in Table 4. In both models, the Ni(II) ion is bound to its ligands in a slightly distorted octahedral geometry (root mean square deviation (RMSD) with respect to the ideal

octahedral geometry of 0.254 and 0.269 Å, respectively, as calculated by the USCF Chimera software [55]). The bond distances are in good agreement with previously reported EXAFS data [27] (Table 4) and do not allow discrimination between the two models ($\chi^2 = 0.051$ and 0.043 for model **1** and **2**, respectively). On the other hand, the orientation of the His2 side-chain, measured considering the dihedral angle formed by the imidazole ring and the Ni(II) ion, is less distorted in model **1**.

Discussion

The structure of wild-type apo,Zn-*HpHypA* at pH 7.2 (Fig. 2) reveals an elongated protein with two quasi-rigid and well-separated metal-binding domains connected by a linker that suggests the possible folding of the Zn-binding domain into closer proximity with the Ni-binding domain. The NMR studies indicate that the two well-ordered domains have a variable relative orientation, even though no evidence supports the possibility for the two domains to fold into a more compact, not-elongated structure that would bring the Ni- and Zn-binding sites close to each other. This NMR structure largely confirms the overall protein structure, also determined by NMR, found for a construct of the apo,Zn-*HpHypA* protein that features a modified N-terminal sequence [31]. While the N-terminal Gly-Ser extension in the modified protein does not perturb the overall structure, it would be expected to alter the protein dynamics of the N-terminus and the structure of the Ni binding site (*vide infra*). Indeed, the modified protein is not functional as a Ni metallochaperone and has an artefactual Ni coordination environment [27].

Molecular dynamics simulations confirmed the small mutual movements of the metal-binding domains seen by NMR, but also revealed motions in the N-terminal Ni binding region. While the flexibility provided by the linker region between the two domains was shown to have no physiological consequence, the motions of the N-terminus correspond to a folding and unfolding of the N-terminal helix that forms the Ni-binding site. The folded structure of the N-terminal helix would be expected to be stabilized in the Ni complex, which cannot properly form in the N-terminally modified protein, providing a mechanism for molecular recognition of the Ni(II) complex, with unwinding pointing toward a possible mechanism for Ni release. This structure may also be important for the interaction between *HpHypA* and *HpUreE* dimers, which has a micromolar dissociation constant in the absence of Ni but binds one Ni(II) ion with nanomolar affinity [24].

Particular attention was paid to examining structural perturbations that occur as a result of pH changes and Ni(II) binding to apo,Zn-*HpHypA* in order to test a model derived from XAS studies that features a change in the Zn coordination from Zn(Cys)₄ to Zn(Cys)₂(His)₂ upon lowering the pH from 7.2 to 6.3 and binding Ni(II) [30]. Only small chemical shift perturbations were observed due to the pH change, indicating that no major rearrangement of the protein structure occurs. Larger perturbations in the NMR spectra are observed upon Ni(II) binding to apo,Zn-*HpHypA*, including the loss of several signals due to paramagnetic line broadening. However, the Zn-binding domain remains relatively unperturbed. Thus, there is no evidence for a pH-induced structural change in the Zn(II) coordination environment from room temperature high-resolution NMR.

The paramagnetic Ni(II) ion causes 12 N-H signals to be broadened beyond detection. These signals correspond to Glu3, Val7, Ser8, Ala38, Met39, Asp40, Lys41, Ser42, Leu43, Phe44, Val45 and Ala47, all of which are in the Ni-binding domain, and include proposed Ni ligands Glu3 and Asp40. A previous study of a non-functional form of apo,Zn-*HpHypA* containing two additional residues at the N-terminus resulted in the artefactual characterization of a diamagnetic Ni(II)-binding site [31]. Another report showed that the wild-type HypA protein contains a paramagnetic Ni(II) center, but identified a somewhat different set of residues with NMR signals obliterated by Ni(II) paramagnetism, including some in the Zn-binding domain [30]. This discrepancy can be explained by an erroneous assignment of the affected NMR resonances derived from the use of a closest-neighbor criterion for signal assignment of the ^1H , ^{15}N HSQC spectrum of Ni,Zn-*HpHypA*, which fails in the presence of slow-exchange between the apo,Zn- and Ni,Zn-forms of the protein, operative in this case. This pitfall has been avoided here by assigning the signals using triple resonance experiments under all four conditions, as described above. Again, little perturbation of the NMR was observed in the Zn-binding domain upon the addition of Ni(II). On this basis, a previously developed model in which the Zn(II) coordination environment was influenced by the presence of Ni(II) at pH 6.3 [30] is not consistent with the high-resolution NMR data obtained in the present study.

The observation and analysis of hyperfine-shifted ^1H -NMR resonances due to the binding of Ni(II) to apo,Zn- *HpHypA* have greatly clarified the structure of the Ni(II) binding site and allowed for a detailed model of the Ni(II) binding site in the protein to be developed. Multiple sequence alignments of HypA homologs reveal an invariant N-terminal MHE sequence [27], where prior mutagenesis studies have identified His2 as a Ni(II) ligand [21]. Based on chemical shift perturbations assigned to the diamagnetic Ni(II) site in N-terminally modified HypA, a planar four-coordinate model with ligands comprised the His2 imidazole and backbone N-donor atoms from His2, Glu3, and Asp40. Mutagenesis coupled with XAS structural analysis of the Ni site identified the N-terminal amine as a Ni(II) ligand [27]. XAS also characterized a six-coordinate Ni site comprised of six N/O-donor ligands, one of which was an imidazole ring, and provided evidence for the coordination of two backbone N-donors in the form of C atoms in the second coordination sphere whose positions were ordered by the formation of a five-membered chelate ring [27]. The analysis of the eight observed hyperfine-shifted ^1H -NMR resonances (*vide supra*) permits the identification of the His2 side-chain imidazole coordinated to Ni(II) by the N δ 1 atom, and a geminal βCH_2 or γCH_2 from the side chains of Met1, His2, Glu3 or Asp40.

Using all the available structural data, computational models of the Ni(II) site structure were developed (Fig. 10). The results of DFT calculations support two models for the structure of the Ni(II) binding site in Ni,Zn-*HpHypA*, in which the nickel ion is bound in a slightly distorted octahedral coordination (Fig. 10). The ligands in the two models are composed of the neutral N-terminal amine group of Met1, the deprotonated amide N atoms of His2 and Glu3, the carboxylate side chains of Glu3 and Asp40 and a His2 imidazole N atom. The two models differ by having the carboxylate group of either Asp40 (model 1) or Glu3 (model 2) *trans* to the imidazole ring of His2. In both models, the N δ 1 of His2 is bound to Ni(II), in agreement with the hyperfine-shifted NMR spectral analysis. The DFT calculations also

indicate that a very large portion of the spin density (ca. 86% in both models) is located on the Ni(II) ion, with the remaining spin density distributed among all other 65 nuclei. Among the latter, the largest spin density (< 3%) is observed for the six N/O atoms bound to Ni(II). This indicates that the NMR relaxation rates are largely dominated by a metal-centered approximation, which validates the use of the Solomon equations for R_1 and R_2 [91] to obtain reliable estimates for metal-to-proton distances. The latter further allows us to use the models to get insights into the assignment of the hyperfine-shifted ^1H NMR signals (Fig. 8; Table 3). Signals A and D, assigned to N ϵ 2H and N δ 2H protons with a distance from Ni(II) estimated as ca. 5.2 Å, are found in the models in the range 5.1–5.4 Å. On this basis, the distances of protons belonging to signals C, H and E, shown to belong to the same spin system, are expected in the range 4.5–5.2 Å, 5.1–5.6 Å, and 6.0–6.7 Å, respectively. Considering the two models, we can exclude His2 and Glu3 aliphatic side chains as responsible for these three signals, while the side chain of Asp40 and Met1 is consistent with these distances in model 1 and 2, respectively.

Signals F and G belong to protons that, according to Eq. (1), are expected to be 5.2–5.9 Å and 4.7–5.3 Å apart from the metal center, respectively. Again, analysis of the two models indicates that the only options are in model B H β or H γ of Met1, while in model 2 they are only consistent with H α and H β of Asp40, i.e., the complementary assignment of signals C, H, and E to protons of Asp40 in model 1 or to protons of Met1 in model 2. Finally, the large linewidth of signal B suggests that it belongs to a proton located at ca. 3.7–4.4 Å, and analysis of both models indicates that the possibilities are the αCH and βCH_2 of His2, the αH and γH of Glu3, the γH of Met1 or the βCH_2 of Asp40. Among these, only αCH of His2 (in both models) and αH of Glu3 (in model 2) possess enough spin densities to justify the ca. 60 ppm hyperfine shift.

Conclusions

The HypA protein from *H. pylori* serves as an important component of both the [Ni,Fe]-hydrogenase and urease maturation pathways in this human pathogen. The results of this detailed study of the structure and function of the *Hp*HypA protein clarify a number of confusing reports in the literature. The structure of apo,Zn-*Hp*HypA that emerges from 2D, 3D and 4D NMR studies is that of an elongated protein with two relatively rigid and well-separated metal-binding domains connected by a linker region. The orientation of the metal-binding domains is variable, reflecting the flexibility of the linker. Altering the flexibility of the linker via Gly-to-Ala substitutions has no physiological ramification for either acid viability of *H. pylori*, or the ability of HypA to serve as a nickel metallochaperone for urease maturation. Chemical shift perturbations observed over the pH range 7.2–6.3, the latter mimicking the internal pH of *H. pylori* under acid shock conditions, did not reveal any significant structural change. Upon binding Ni(II) several ^1H -NMR resonances are lost from the spectra due to paramagnetic line broadening in the vicinity of the Ni(II) binding site. The analysis of observed hyperfine-shifted ^1H -NMR resonances leads to two closely related models for the structure of the Ni(II) site. These structures consist of six-coordinate Ni(II) sites with coordinating ligands comprising the N-terminal amine, the His2 and Glu3 amide N atoms, the His2 imidazole side chain bound via N δ 1, and the carboxylate side chains of Glu3 and Asp40.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
a ^1H , ^{15}N HSQC spectrum of apo,Zn-HpHypA at 900 MHz at pH 7.2; **b** ^1H , ^{15}N HSQC spectrum of apo,Zn-HpHypA at 900 MHz at pH 7.2 (blue) overlapped with the spectrum of Ni,Zn-HpHypA (red). The spectra show the relative positions of the peaks, each representing an N-H pair of the amides in the protein main chain. Assignments of individual peaks are shown as blue (for apo,Zn-HpHypA) and red (for Ni,Zn-HpHypA) labels containing the single letter amino acid code and the corresponding residue number. The peak positions are relative to the ^1H and ^{15}N chemical shift scales of the ω_1 and ω_2 dimensions, respectively

(ppm scales). The pairs of signals corresponding to the amide groups of glutamine/asparagine residues are also shown, with lines linking the HD21/HE21 (left peak) and the HD22/HE22 (right peak) proton signals

Fig. 2.

Solution structure of apo,Zn-*HpHypA* at pH 7.2. **a** Ribbon diagram of the closest-to-average conformer in the ensemble of the 20 lowest-energy NMR structures, colored from blue in the proximity of the N-terminal to red at the C-terminus; the Zn(II) ion is shown as a blue sphere, coordinated to four thiolate groups of Cys74, Cys 77, Cys91 and Cys94; **b** ribbon diagram of the structural ensemble of the 20 lowest-energy NMR structures, superimposed on the Ni-binding domain constituted by the three-strand β -sheet and the two α -helices; **c** “sausage” representation of the NMR ensemble of apo,Zn-*HpHypA*, colored by secondary

structure elements, with the radius proportional to the RMSD of the Ca atoms; **d** topological diagram of the structure of apo,Zn-*Hp*HypA colored as in **a**

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Fig. 3. Quasi-rigid domain decomposition of apo,Zn-*HpHypA* trajectory. **a** SPECTRUS quality score profile. **b** Domain subdivision of the protein sequence as a function of the number of domains. **c** Ribbon representation of the most representative structure of the most populated cluster colored according to the optimal domain decomposition into four domains reported in **b**. **d** Lys82(N ϵ)-Glu71(C δ) distance variation along simulation time. The gray line represents the effective sampling of the distance during the simulation, while the black line is obtained by applying a Fast Fourier Transform filter to reduce the noise

Fig. 4.

The HypA mutant strains are not attenuated for acid survival. The wild-type (WT) strain, urease mutant strain (*ureB*), HypA restorant (*hypA*-restorant) and HypA mutant strains (*hypA::kansacB*, G32A, G89A, G32A/G89A, G34A, G104A, and G34A/G104A) were incubated for 1 h in PBS adjusted to pH 6.0 (**a**, **b**) or pH 2.3 (**c**, **d**), in the absence (**a**, **c**) or presence (**b**, **d**) of 5 mM urea. Colony forming units (CFU) were enumerated at 0 min (T_0) and 60 min (T_{60}). Two sets of experiments were performed, denoted by the gap and double line in the *x*-axis, with HypA mutants G32A, G89A, and G32A/G89A tested in the first set, and HypA mutants G34A, G104A, and G34A/G104A tested in the second set. The controls (WT, *ureB*, *hypA::kan-sacB*, and *hypA*-restorant) were performed with each set of experiments. Percent survival was calculated as $\text{CFU at } T_{60} / \text{CFU at } T_0 \times 100$. Data from individual replicates are shown as points, with the bar plotted at the geometric mean. Open symbols indicate that no bacteria were recovered at T_{60} and are plotted as a function of the limit of detection (100 or 500 CFU/mL for the first and second set of experiments, respectively). Three biological replicates were performed. In **a–c**, a one-way ANOVA

followed by Dunnett's test for multiple comparisons was performed; the comparisons were made only to WT. In **d**, the same statistical tests were performed on the log-transformed data. ** $p < 0.01$; **** $p < 0.0001$

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Fig. 5. Chemical shift perturbations (CSP, ppm) obtained by comparing apo,Zn-*HpHypA* at pH 7.2 and pH 6.3 (H,N in **a**; C α ,C β in **b**), apo,Zn-*HpHypA* and Ni,Zn-*HpHypA* at pH 7.2 (H,N in **c**; C α ,C β in **d**), apo,Zn-*HpHypA* and Ni,Zn-*HpHypA* at pH 6.3 (H,N in **e**; C α ,C β in **f**), and Ni,Zn-*HpHypA* at pH 7.2 and pH 6.3 (H,N in **g**; C α ,C β in **h**). Blue bars indicate the residues comprising the Zn-binding domain

Fig. 6. Comparison of the ^1H , ^{15}N HSQC spectra for Cys74, Cys 77, His79, Cys91, Cys94 and His95 as a function of pH and Ni(II)-binding. Color code: apo,Zn-*HpHypA* at pH 7.2 = cyan; Ni,Zn-*HpHypA* at pH 7.2 = blue; apo,Zn-*HpHypA* at pH 6.3 = orange; Ni,Zn-*HpHypA* at pH 6.3 = red

Fig. 7.

Ribbon diagram of the closest-to-average conformer of the NMR structure ensemble of apo,Zn-*HpHypA* at pH 7.2, highlighting in red the residues for which the amide NH signals are broadened beyond detection upon binding of paramagnetic Ni(II). The Zn(II) ion is shown as a violet sphere, coordinated by the four thiolate groups of Cys74, Cys 77, Cys91 and Cys94. The side chains of the putative Ni(II)-binding residues Met1, His2, Glu3 and Asp40 are also shown

Fig. 8.
400 MHz ^1H NMR spectra of Ni,Zn-*HpHypA* as a function of pH and presence of deuterated water, at 298 K

Fig. 9. 400 MHz ^1H NMR NOE difference spectra of Ni,Zn-*HpHypA* obtained upon saturation of signals A (red trace), C (blue trace), D (green trace) and H (violet trace); the 1D non-saturated ^1H spectrum is reported in the top trace (black). The signals connected through dipolar interactions are shown

Fig. 10. B3LYP/G 6—311(*p,d*) optimized geometries of model **1** and **2** for the Ni(II)-binding site in Ni,Zn-*HpHypA*

Table 1Structural statistics and restraints for solution structure of apo,Zn-*HpHypA* at pH 7.2

Number of restraints^a	
Total NOE restraints	2694
Intra-residual NOE restraints ($ i-j = 0$)	1320
Sequential NOE restraints ($ i-j = 1$)	395
Medium range NOE restraints ($1 < i-j < 5$)	294
Long-range NOE restraints ($ i-j > 4$)	620
Ambiguous NOE restraints	65
Average number of NOE restraints per residue	23.03
Average number of long-range NOE restraints per residue	5.30
Dihedral angle restraints	191
Restraints violations	
Average number of NOE violations $> 0.5 \text{ \AA}$	0 ± 0.0
RMS of NOE violations ($\text{\AA}$)	0.047 ± 0.003
Average number of dihedral angle violations $> 5^\circ$	0.05 ± 0.22
RMS dihedral angle violations ($^\circ$)	0.203 ± 0.104
Deviation from ideal geometry	
RMSD from ideal geometry for bond lengths ($\text{\AA}$)	0.019 ± 0.001
RMSD from ideal geometry for bond angles ($^\circ$)	1.808 ± 0.061
Ramachandran statistics from Procheck	
Favored regions (%)	90.9 ± 1.1
Allowed regions (%)	9.0 ± 1.2
RMSD from mean coordinates	
Backbone atoms (range 1–117) ($\text{\AA}$)	0.78 ± 0.18
Heavy atoms (range 1–117) ($\text{\AA}$)	1.02 ± 0.16
Backbone atoms (Ni-domain, range 1–68, 107–117) ($\text{\AA}$)	0.35 ± 0.08
Heavy atoms (Ni-domain, range 1–68, 107–117) ($\text{\AA}$)	0.68 ± 0.10
Backbone atoms (Zn-domain range 71–103) ($\text{\AA}$)	0.39 ± 0.10
Heavy atoms (Zn-domain range 71–103) ($\text{\AA}$)	0.79 ± 0.16
Global quality scores	
MolProbity Clashescore	1.76 ± 1.01
WHATIF packing quality Z-score	-0.76 ± 0.08
WHATIF backbone normality Z-score	-1.34 ± 0.30
WHATIF Chi1/Chi2 rotamer normality Z-score	1.06 ± 0.41
WHATIF Ramachandran plot Z-score	0.38 ± 0.47

^aInput generated in the final iteration of the ARIA/YASARA structure calculation [46, 47]. The entries refer to the 20 best YARIA conformers after energy minimization with YASARA [47]. Where applicable, the average value for the bundle of 20 conformers and the standard deviation are given

Table 2

^{13}C chemical shifts of selected cysteine and histidine residues of apo.Zn- and Ni.Zn-*HpHypA* as a function of pH

	N						C α						C β					
	Apo.Zn			Ni.Zn			Apo.Zn			Ni.Zn			Apo.Zn			Ni.Zn		
	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2	pH 6.3	pH 7.2
H																		
Cys74	9.22	9.28	9.19	9.28	128.26	128.29	128.34	128.27	128.34	59.21	59.27	59.27	59.27	59.27	32.39	32.38	32.32	32.28
Cys77	9.76	9.80	9.75	9.80	123.73	123.92	123.97	123.66	123.97	58.96	58.95	58.95	59.10	59.10	32.16	32.17	32.20	32.10
His79	8.84	8.85	8.84	8.85	124.77	125.28	125.29	124.52	125.29	58.03	58.23	58.04	58.23	58.23	32.48	32.92	32.38	32.81
Cys91	9.00	9.00	9.00	9.01	126.00	126.04	126.09	126.00	126.09	60.16	60.30	60.53	60.51	60.51	31.04	31.08	30.95	30.95
Cys94	8.97	8.99	8.96	8.99	120.47	120.60	120.67	120.44	120.67	58.67	58.58	58.76	58.63	58.63	31.87	31.94	31.77	31.88
His95	7.63	7.47	7.50	7.48	116.17	116.57	116.53	115.92	116.53	56.95	57.49	57.09	57.49	57.49	26.17	27.05	26.09	26.63

Table 3
Spectroscopic parameters for the paramagnetic ^1H NMR signals of Ni₂Zn-*HpHypA*

Signal	δ (ppm) @ 298 K	T_1 (ms) @ 400 MHz	ν (kHz) @ 400 MHz	Assignment (model 1/model 2)
A	66.4	0.91 ± 0.03	0.60 ± 0.06	His2 HNe2
B	59	0.15 ± 0.19	2.20 ± 0.22	His2 H α or Glu3 H α
C	43.8	0.74 ± 0.04	0.82 ± 0.08	Asp40 H β /Met1 H β
D	38.1	1.39 ± 0.02	0.41 ± 0.04	His2 HN62
E	-1.51	4.00 ± 0.26	0.16 ± 0.02	Asp40 H α /Met1 H γ
F	-2.88	0.87 ± 0.18	0.34 ± 0.03	Met1 H β , γ /Asp40 H α
G	-7.7	0.57 ± 0.05	0.67 ± 0.07	Met1 H γ /Asp40 H β
H	-19.3	1.56 ± 0.02	0.48 ± 0.05	Asp40 H β /Met1 H β

Table 4
Selected distances, angles and dihedrals around the Ni(II) ions in optimized models **1** and **2**

Parameter	Model 1	Model 2	Experimental ^a
Ni(II)—Met1(N)	2.185	2.179	2.21
Ni(II)—His2(N)	1.978	2.005	1.92
Ni(II)—His2(Nδ1)	2.263	2.272	2.08
Ni(II)—Glu3(N)	2.079	2.048	1.97
Ni(II)—Glu3(Oε1)	2.016	2.117	2.08
Ni(II)—Asp40(Oδ1)	2.231	2.093	2.08
Met1(N)—Ni(II)—His2(N)	79.0	79.3	
Met1(N)—Ni(II)—His2(Nδ1)	98.1	97.4	
Met1(N)—Ni(II)—Glu3(Oε1)	89.7	87.7	
Met1(N)—Ni(II)—Asp40(Oδ1)	75.4	95.2	
Glu3(N)—Ni(II)—His2(N)	83.2	80.1	
Glu3(N)—Ni(II)—His2(Nδ1)	85.3	83.6	
Glu3(N)—Ni(II)—Glu3(Oε1)	109.1	94.5	
Glu3(N)—Ni(II)—Asp40(Oδ1)	99.5	105.9	
His2(N)—Ni(II)—His2(Nδ1)	81.4	84.0	
His2(N)—Ni(II)—Asp40(Oδ1)	93.2	164.4	
Glu3(Oε1)—Ni(II)—His2(Nδ1)	85.5	170.4	
Glu3(Oε1)—Ni(II)—Asp40(Oδ1)	98.4	89.3	
Met1(N)—Ni(II)—Glu3(N)	161.1	159.1	
His2(N)—Ni(II)—Glu3(Oε1)	161.3	104.9	
His2(Nδ1)—Ni(II)—Asp40(Oδ1)	172.3	82.2	
Ni(II)—His2(Nδ1)—His2(Cε1)—His2(Nε2)	154.5	146.8	
Ni(II)—His2(Nδ1)—His2(Cγ)—His2(Cδ2)	−154.8	−145.3	

Distances are in Angstroms, while angles and dihedrals are in degrees

^a Taken from Ref. [27]