Helicobacter pylori UreE, a urease accessory protein: specific Ni\(^{2+}\)- and Zn\(^{2+}\)-binding properties and interaction with its cognate UreG

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INTRODUCTION

Helicobacter pylori is a widespread human pathogen that colonizes the gastric mucosa of approx. 50% of the world human population [1] and is responsible for severe diseases such as chronic gastritis, peptic and duodenal ulcers that eventually may lead to cancer [2]. The bacterium is able to survive in the hostile environment of the human stomach through the activity of urease.

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Key words: accessory protein UreE, accessory protein UreG, Helicobacter pylori urease assembly, nickel trafficking, protein–protein complex formation, zinc binding.

Abbreviations used: Bp, Bacillus pasteurii; Hp, Helicobacter pylori; HSQC, heteronuclear single quantum correlation; ITC, isothermal titration calorimetry; Ka, Klebsiella aerogenes; MALD, multiple angle light scattering; Mj, Methanocaldococcus jannaschii; GELS, quasi-elastic light scattering; SEC, size-exclusion chromatography; TROSY, transverse relaxation optimized spectroscopy.

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The nucleotide sequence data reported in this study will appear in the DDBJ, EMBL, GenBank®, and GSDB Nucleotide Sequence Databases under the accession number ABM16833.
UreE [20]) and of a truncated form of KaUreE (Klebsiella aerogenes UreE), H144*KaUreE [21], indicate that UreE are symmetric homodimers, with each monomer made of an N-terminal domain and a C-terminal domain, the latter involved in the head-to-head dimerization. A metal-ion-binding site, found at the protein dimerization interface, involves two conserved histidines, one from each monomer (His100 in BpUreE and His96 in KaUreE, Figure 1A). This site contains Zn$^{2+}$ in the structure of BpUreE and Cu$^{2+}$ in that of H144*KaUreE, but is generally assumed to be occupied by Ni$^{2+}$ in the protein functional form in vivo, a hypothesis supported by anomalous difference X-ray diffraction maps of BpUreE crystals soaked in a Ni$^{2+}$ solution [20]. The structure of H144*KaUreE also contains two additional Cu$^{2+}$ ions bound to a pair of histidines on the surface of each monomer (His101 and His112), but these residues are not conserved in BpUreE (Tyr114 and Lys116), HpUreE (H. pylori UreE) (Phe118 and Lys120) (Figure 1) or other sources [22].

An alternative possible physiological role for UreE is suggested by the observation that the GTP concentration needed for optimal activation of urease in vitro is greatly reduced in the presence of UreE as compared with that required in its absence [19]. This implies that UreE must play an important direct or indirect role in the functional activation of UreG. In this respect, in vivo studies using yeast two-hybrid analysis [23,24] as well as co-immunoprecipitation assays [24] indicated a direct interaction between UreE and UreG from H. pylori.

On the basis of the available evidence, the question of which functional role is played by UreE in the urease active site assembly is still awaiting a definitive answer. The present study represents an attempt to clarify some details of the reactivity of UreE towards Ni$^{2+}$ and Zn$^{2+}$, as well as with HpUreG.

**EXPERIMENTAL**

**Protein preparations**

Recombinant wild-type HpUreE was prepared using a protocol adapted from a previous study [25]. Purity was checked...
using SDS/PAGE (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/422/bj4220091add.htm). Site-directed mutagenesis, aimed at the production of the H102K and H152A mutants, was carried out using standard procedures. The full experimental details of gene cloning, protein expression and purification are provided in the Supplementary Online Data (http://www.BiochemJ.org/bj/422/bj4220091add.htm). Recombinant HpUreG and its C66A/H68A mutant were obtained as previously described in [16].

CD spectroscopy

The secondary structure of HpUreE was evaluated by CD spectroscopy, performed on the protein (5 μM) diluted in 20 mM phosphate buffer, pH 8.0, using a Jasco 780 810 spectropolarimeter flushed with N₂, and a cuvette with 0.1 cm path-length. Ten spectra were accumulated from 190 to 240 nm at 0.2 nm intervals, using the same buffer containing 20 μM MgCl₂ and 150 mM NaCl, and eluted at a flow rate of 0.6 ml/min. The concentration of the eluted protein was determined using a refractive index detector (Optilab DSP, Wyatt). The solvent refractive index. Molecular masses were determined from Light scattering measurements

In a typical experiment, a protein sample (100 μl, 50 μM) was loaded on to a size-exclusion Superdex 200 HR 10/30 column (GE Healthcare), pre-equilibrated using 20 mM Tris/HCl, pH 8.0, and 150 mM NaCl, and eluted at a flow rate of 0.6 ml/min. The column was connected downstream to a multi-angle laser light (690.0 nm) scattering DAWN EOS photometer (Wyatt Technology) and to a quasi-elastic light scattering WyattQELS device. The concentration of the eluted protein was determined using a refractive index detector (Optilab DSP, Wyatt). The specific refractive index increment (dn/dc) for the proteins was taken as 0.185 ml/g [28]. The value of 1.321 was used for the solvent refractive index. Molecular masses were determined from a Zimm plot. Data were recorded and processed using the Astra 5.1.9 software (Wyatt Technology), following the manufacturer’s indications. When the measurements were carried out in the presence of metal ions, stoichiometric amounts of ZnSO₄ or NiSO₄ were added to the protein samples before loading it on to the size-exclusion column, and the protein was eluted using the same buffer containing 20 μM ZnSO₄ or NiSO₄. In order to explore the formation of a HpUreE–HpUreG interaction, a solution containing HpUreE (50 μM dimer) and HpUreG (100 μM monomer) was analysed in the absence and in the presence of 200 μM NiSO₄ or 200 μM ZnSO₄ under the same experimental conditions.

ITC (isothermal titration calorimetry) experiments

Titration experiments were performed at 25°C using a high-sensitivity VP-ITC microcalorimeter (MicroCal LLC, Northampton, MA, U.S.A.). The proteins and the metal ions (from 100 mM stock solutions) were diluted using the same buffer (20 mM Tris/HCl, pH 7.0, and 150 mM NaCl) and eluted from a size-exclusion column, utilized immediately before the ITC measurement to freshly purify the protein. The measuring cell contained 1.409 ml of protein solution, and the reference cell was filled with deionized water. Before starting the experiments, the baseline stability was verified. A spacing of 400–600 s between injections was applied in order to allow the system to reach thermal equilibrium after each addition. For each titration, a control experiment was carried out by adding the titrating solution into the buffer alone, under identical conditions. Heats of dilution were negligible. In a set of experiments aimed at determining the metal-binding properties of HpUreE, the protein (10 μM) or its H102K and H152A mutants (10 μM) were titrated with 30 injections (10 μl each) of a solution containing 100 μM NiSO₄ or ZnSO₄. In order to determine the binding parameters of HpUreE to HpUreG, the latter protein (50 μM monomer) was titrated with 30 injections (10 μl each) of a solution containing 160 μM HpUreE dimer in the same buffer. In the case of Zn²⁺ titration on to the HpUreE–HpUreG complex (5 μM), generated in situ by mixing 5 μM HpUreE and 10 μM HpUreG monomer, 30 aliquots (10 μl each) of a solution containing 70 μM ZnSO₄ were injected into the protein solution. Identical setup was used for the related mutants. The details of data analysis are given in the Supplementary Online Data. The dissociation constants and thermodynamic parameters provided in the present study do not take into account possible events of proton transfer linked to metal binding, or the presence, in solution, of complexes between the metal ions and the buffer. This treatment is beyond the scope of the present study. However, the values of the measured equilibrium constants compare well with those reported in the literature and determined using ITC or other methodologies such as equilibrium dialysis coupled to metal analysis, which, in principle, should also take into account similar effects. These values are therefore only used for comparison purposes.

Structural modelling of the HpUreE–HpUreG complex

The alignment of HpUreE to HpUreG [22] was used to calculate, using the MODELLER9v5 software [29], 50 structural models of the HpUreE dimer, imposing the structural identity of the two monomers. The best model was selected on the basis of the lowest value of the MODELLER objective function. The ROSETTADOCK software [30] was used to calculate an initial complex between the model structure of dimeric HpUreG [16], and the central C-terminal domains of dimeric HpUreE. The complex with the best ROSETTADOCK score was selected among all generated models for the subsequent refining run, carried out by applying 1000 times a perturbation to the starting structure. The Cα trace of this complex was used, together with the crystal structures of MjHyPB (Methanocaldococcus jannaschii HypB [31], PDB code 1HF8), BpUreE [20] (PDB code 1EAR), and KuUreE [21] (PDB code 1GMW) as templates to build 200 structural models of the HpUreE–HpUreG complex using the MODELLER9v5 software [29]. The best model was selected on the basis of the lowest value of the MODELLER objective.
A prediction of the secondary structure elements of HpUreE (Figure 1B) based on the Jpred algorithm [32] indicates 21% α-helix and 23% β-strand content, with the remaining 56% constituting turns or random coil conformations. Figure 1(B) also suggests that a similar fold is attained by several different UreE proteins, as previously proposed on the basis of modelling studies [22]. The CD spectrum of HpUreE (Figure 2A) shows the presence of both α-helices and β-strands, with negative deflections around 218 nm and 208 nm and a positive peak at 190 nm. The CD spectrum was quantitatively analysed and the best fit [NRMSD (normalized root mean square deviation) = 0.029] estimated a secondary structure composition of 13% α-helix, 33% β-strand, 23% turns and 30% random coil for HpUreE. This composition is similar to that calculated using the DSSP program [33] for the crystallographic structures of Zn2+-bound BpUreE [20] (18% α-helix, 37% β-sheet) and of the Cu2+-bound H144*KaUreE [21] (13% α-helix, 25% β-sheet). The 1H chemical shift spreading observed in the TROSY–HSQC NMR spectrum (Figure 2B) ranges from 6.5 to 10 p.p.m., as observed in the 1H,15N HSQC NMR spectrum of BpUreE [34], suggesting similar extent of fold for the two proteins.

The molecular mass and the hydrodynamic radius of HpUreE in solution were determined using a combination of SEC (size-exclusion chromatography) and light scattering [MALS (multiple angle light scattering)/QELS (quasi-elastic light scattering)] (Figure 2C). The elution profile and the light scattering data show that HpUreE is a dimer in solution with M = 43.1 ± 4.8 kDa and Rg = 3.0 ± 1.4 nm (theoretical mass = 39 kDa). This is consistent with all available crystallographic structural information on UreE proteins (Figure 1B) [20,21] as well as with previous evidence collected on HpUreE based on SEC criteria [25]. The light scattering measurements exclude the possibility that oligomers of the apo-protein are formed in solution for concentrations lower than 50 µM, as instead previously proposed for H144*KaUreE [35]. The better quality of the 1H,15N TROSY–HSQC (Figure 2B) with respect to the simple 1H,15N HSQC experiment (results not shown) further supports the presence of a dimeric form at 0.1–0.3 mM concentration. The symmetric architectural arrangement of the two monomers is revealed by the number of observed peptide NH peaks in the NMR spectrum: about 120 unique peaks, out of the expected 170 residues per monomer, can be observed, with missing signals probably including the C-terminal 30 residues predicted to be unstructured using Jpred (Figure 1B).

In particular, in the case of glycine residues, seven glycines are present in the sequence of HpUreE (Figure 1B) and the same number of peaks is observed in the 15N 100–110 p.p.m. range typical for glycine NH signals (Figure 2B).

**HpUreE metal-binding properties**

Ni2+ is generally considered to be the physiological cofactor of UreE, and the understanding of the structural features of Ni2+ binding is therefore important to clarify the role of this chaperone in vivo. Moreover, in several recent instances, interplay between Ni2+ and Zn2+ has been observed and proposed to be functionally important in regulating cellular trafficking of metal ions [13–16,31,36–40]. In particular, Zn2+ is involved in the dimerization of HpUreG, a process that plays a potential regulatory role in the urease active site assembly [16]. Previous equilibrium dialysis experiments carried out on HpUreE established a 1:1 stoichiometry for the Ni2+ binding to the homodimeric protein, with Kd approx. 1 µM [25]. However, the experiments were carried out at pH 8.25 in an apparently non-buffered solution containing only NaCl, no thermodynamic parameters for the metal binding event were determined, and the binding affinity for Zn2+ was not measured [25]. In the present study, the Ni2+ binding to HpUreE was investigated using ITC, and a comparison between Ni2+ and Zn2+ binding was performed.
The ITC measurements were carried out by adding Ni\textsuperscript{2+} or Zn\textsuperscript{2+} to the apo-protein in a buffered solution at pH 7.0, and the occurrence of a binding event was revealed by the presence of exothermic peaks that followed each addition (Supplementary Figures S2A and S2B at http://www.BiochemJ.org/bj/422/bj4220091add.htm for Ni\textsuperscript{2+} and Zn\textsuperscript{2+} respectively). Fits of the integrated heat data (Figure 3A and 3B) for Ni\textsuperscript{2+} and Zn\textsuperscript{2+} respectively) were carried out using the simplest model, which entails a single binding event, and yielded a stoichiometry of one equivalent of Ni\textsuperscript{2+} or Zn\textsuperscript{2+} bound to the HpUreE dimer. Dissociation constants $K_\text{d}(\text{Ni}) = 0.15 \pm 0.01 \mu M$ and $K_\text{d}(\text{Zn}) = 0.49 \pm 0.01 \mu M$ were calculated for Ni\textsuperscript{2+} and Zn\textsuperscript{2+} binding respectively. In both cases, these processes are driven by favourable enthalpic factors $\Delta H(\text{Ni}) = -13 \pm 1 \text{ kcal/mol}$ (1 cal=4.184 J), $\Delta H(\text{Zn}) = -10 \pm 1 \text{ kcal/mol}$ that compensate the negative entropic values $\Delta S(\text{Ni}) = -13 \text{ cal mol}^{-1} \text{ K}^{-1}$, $\Delta S(\text{Zn}) = -4 \text{ cal mol}^{-1} \text{ K}^{-1}$ calculated from the fit. The values of the $K_\text{d}(\text{Ni})$ and $K_\text{d}(\text{Zn})$ measured for HpUreE are comparable to those established by ITC for the binding of Ni\textsuperscript{2+} and Zn\textsuperscript{2+} to BpUreE and H144* HpUreE [35], and by equilibrium dialysis for the binding of Ni\textsuperscript{2+} to BpUreE [39], of Ni\textsuperscript{2+} and Zn\textsuperscript{2+} to H144* HpUreE [41,42] and of Ni\textsuperscript{2+} to HpUreE [25]. All these values are consistent with a role of intracellular metal ion transport associated with UreE proteins [43].

In the structure of Zn\textsuperscript{2+}-BpUreE [20] and Cu\textsuperscript{2+}-H144* HpUreE [21], the metal ions are bound to the surface of the protein using the conserved His\textsuperscript{100} and His\textsuperscript{96} residues respectively (Figure 1A). In order to firmly establish the role of the corresponding His\textsuperscript{100} in the binding of Ni\textsuperscript{2+} and Zn\textsuperscript{2+} to HpUreE, the H102K mutant was obtained by site-directed mutagenesis. ITC titrations of H102K HpUreE with Ni\textsuperscript{2+} and Zn\textsuperscript{2+}, performed under identical conditions as for the wild-type protein, proved the absence of a binding event (Figures 3A and 3B), confirming the key role of this residue in metal binding to HpUreE.

In the crystal structure of Zn\textsuperscript{2+}-bound BpUreE, the protein is present as a dimer of dimers, with the metal ion in a bridging position, bound to four conserved His\textsuperscript{100} residues, one from each monomer [20]. However, this oligomerization has been observed for BpUreE only in the solid state [20] or in concentrated (mM) solutions [34], while dynamic light scattering of the metal-bound protein in the 50–250 μM range excluded this effect [39]. The molecular mass and hydrodynamic radius of BpUreE (50 μM dimer) in the absence and in the presence of two equivalents of Ni\textsuperscript{2+} and Zn\textsuperscript{2+} per dimer were determined in the present study using SEC on-line with MALS and QELS. The results indicate that, like HpUreE, BpUreE is a dimer in solution both in the absence and in the presence of these metal ions, with $M = 41.0$ kDa and $R_h = 3.0$ nm.] In the case of HpUreE, the influence of metal binding on the quaternary structure of the protein in the range of concentrations used in the microcalorimetric metal binding studies (10–20 μM) was investigated using a combination of light scattering methods (MALS and QELS). The values measured for Ni\textsuperscript{2+}-HpUreE ($M = 45.7 \pm 5.1$ kDa, $R_h = 3.4 \pm 1.5$ nm) and Zn\textsuperscript{2+}-HpUreE ($M = 46.4 \pm 5.2$ kDa, $R_h = 3.3 \pm 1.5$ nm) are similar to those established for the apo-protein (Figure 2C), demonstrating that the metal-bound protein is a dimer, and not a dimer of dimers, independently of the presence of bound metal ions. This is consistent also with the similar linewidths in the TROSY–HSQC NMR spectra of apo-, Ni\textsuperscript{2+}-bound and Zn\textsuperscript{2+}-bound HpUreE, which indicates similar protein size in the various metal-bound states (Supplementary Figure S3 at http://www.BiochemJ.org/bj/422/bj4220091add.htm).

While the dissociation constants observed for the metal ion complexes of HpUreE are in the μM range previously determined for UreE proteins from different sources, the 1:1 metal ion binding stoichiometry established for HpUreE differs from previous data obtained for KaUreE and BpUreE, which indicated 2:1 stoichiometry. KaUreE additionally binds three Ni\textsuperscript{2+} ions to a histidine-rich tail containing ten histidines among the last 15 residues, absent both in HpUreE and in BpUreE [35]. In the case of BpUreE, the presence of a binuclear [Ni(OH)Ni\textsuperscript{2+}] centre was proposed on the basis of EXAFS spectra, rendering this protein not only a Ni\textsuperscript{2+} transporter but also a potential scaffold for the assembly of the dinuclear active site of urease [39]. In BpUreE and KaUreE, a conserved HXH motif is present in this protein region: in particular, in BpUreE the HQH motif is located at the end of the sequence, whereas in KaUreE several possible HXXH concatenated motifs constitute the His-rich tail (HGGHHHAAAHHHHASHH). On the other hand, in HpUreE a single histidine (His\textsuperscript{152}) is observed in the C-terminal tail (Figure 1B). On the basis of these considerations, a possible reason for the different stoichiometry of the Ni\textsuperscript{2+} binding to HpUreE on one side (1:1), and to BpUreE and KaUreE on the other (2:1), might reside in the different sequence motifs of histidine residues found at the C-terminal tails of these proteins. These observations suggest a specialized role, in metal ion storage and/or delivery, for the C-terminal portion of the protein, depending on its length and composition, and prompted us to investigate whether His\textsuperscript{152} is involved in metal ion binding to HpUreE using the H152A mutant.

ITC titrations of H152A HpUreE with Ni\textsuperscript{2+}, performed under identical conditions as for the wild-type protein, proved the occurrence of a binding event (Supplementary Figure S2C). The integrated heat data, fitted using a single site model (Figure 3C), yielded values for the dissociation constant $K_d(\text{Ni}) = 0.87 \pm$
0.01 μM), reaction enthalpy \(\Delta H(Ni) = -12 \pm 1 \text{ kcal/mol}\) and reaction entropy \(\Delta S(Ni) = -11 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}\), which are consistent with those obtained for the wild-type protein (Figure 3A). These data indicate that the His\text{152} residue is not involved in binding the Ni\text{2+} ion in HpUreE, suggesting that this residue is not essential for the nickel-delivery function. In turn, this also supports the concept that both histidine residues found in the HXH motif at the C-terminal end of BpUreE and KaUreE are necessary to build up the dinuclear Ni\text{2+} centre observed in those cases.

On the other hand, the titration of H152A HpUreE with Zn\text{2+} showed clear differences in the binding mode as compared with the wild-type protein (Figure 3D and Supplementary Figure S2D). Best fits of the integrated heat data could be obtained using a model involving not one, as in the case of wild-type HpUreE, but two independent binding events, yielding dissociation constants \(K_{d}(Zn) = 0.13 \pm 0.02 \mu M\) and \(K_{d}(Zn) = 0.82 \pm 0.01 \mu M\). Both events are driven by favourable enthalpic \(\Delta H(Zn) = -4 \pm 1 \text{ kcal/mol}\), \(\Delta H(Zn) = 7 \pm 1 \text{ kcal/mol}\) and entropic \(\Delta S(Zn) = 19 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}\), \(\Delta S(Zn) = 30 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}\) factors. The observation of an additional Zn\text{2+}-binding site upon replacement of a histidine with a non-co-ordinating residue like alanine, could be, at first sight, bewildering. This apparent incongruity, resulting from our experimental data, can be explained by taking into consideration the peculiarity of the protein region where the mutation is carried out. This is a long extended and flexible stretch whose conformational or relative orientation with respect to the rest of the protein could change as a consequence of point mutations. Therefore, it is possible that, while His\text{152} is involved in Zn\text{2+} binding by isolated wild-type HpUreE, the resulting conformation of the flexible C-terminal arm masks an additional binding site, which becomes accessible upon mutation of this residue.

The conclusions that can be drawn from these results are that Ni\text{2+} and Zn\text{2+} bind to wild-type HpUreE using different modes. Although Ni\text{2+} is bound to the conserved His\text{102} on the surface of the dimer, without any involvement of His\text{152}, Zn\text{2+} binding not only requires the His\text{102} pair, but is also modulated by the two His\text{152} residues at the C-terminal position. A different binding mode for the two metal ions to the wild-type protein is supported by a comparison of the TROSY–HSQC spectra of HpUreE in the apo-form with the same spectra of the Zn\text{2+} and Ni\text{2+}-bound forms (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/422/bj20091add.htm). Residues changing their chemical shifts upon nickel addition are also affected (and at the same extent) by the presence of Zn\text{2+}. In addition, a few more peaks change their position in the presence of Zn\text{2+}, consistently with the involvement of a larger number of residues in the Zn\text{2+}-binding event. The higher availability of intracellular Zn\text{2+} as compared with Ni\text{2+}, together with the similar affinity of HpUreE for these two metal ions described above, suggests that the specificity of binding different metals must rely on changes in ligand environment, as observed experimentally.

At least one histidine residue is always present near the C-termini of all UreE sequences [22], although this feature is not maintained in the H152A HpUreE mutant, or in H144*KaUreE (Figure 1B). Consistent with this observation, the calorimetric Zn\text{2+} titration curve obtained for H152A HpUreE resembles the one reported for the binding of Ni\text{2+} and Cu\text{2+} to H144*KaUreE at protein concentrations \(\geq 25 \mu M\) [35]. The data for the latter protein were interpreted as indicating an initial binding of two Ni\text{2+} or Cu\text{2+} ions, one after the other, to the interface of the H144*KaUreE dimer of dimers, assumed to be the most abundant species in solution. This binding site was suggested to give rise to a tetrameric structure and to involve the four conserved His\text{96} residues (one per each monomer) [35]. In the case of HpUreE, however, no oligomerization events occur for the H152A mutant in the presence of Zn\text{2+} or Ni\text{2+}, as demonstrated using light scattering experiments (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/422/bj20091add.htm).

HpUreE–HpUreG interaction

The available experimental evidence indicate that UreE and UreG form a functional complex in vitro [19,23,24]. In order to observe and characterize this interaction in vitro, a solution containing equimolar amounts of the two purified apo-proteins was analysed by SEC and light scattering (Figure 4A). The result of this experiment indicates that the dimeric HpUreE elutes as a species separated from HpUreG, the latter being present in the monomeric state, as recently reported [16]. Considering that the experimental setup used for the SEC–MALS–QELS measurement represents non-equilibrium conditions, we monitored this interaction more quantitatively using ITC. When a solution of HpUreE was titrated with a solution of HpUreE in the same buffer, clear exothermic peaks were observed (Supplementary Figure S5A at http://www.BiochemJ.org/bj/422/bj20091add.htm) which, after integration, revealed a curve (Figure 4B) that could be fitted using a single binding event model. The stoichiometry of the interaction suggests that two monomers of HpUreE bind to a single dimer of HpUreE, forming a HpUreE–HpUreG complex having a dissociation constant \(K_{d} = 4.0 \pm 0.5 \mu M\), \(\Delta H = -12.5 \pm 0.9 \text{ kcal/mol}\), and \(\Delta S = -17.5 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}\).

The formation of the HpUreE–HpUreG complex was also monitored using NMR spectroscopy. The TROSY–HSQC spectrum of the solution containing one equivalent of \(^{15}\text{N}-\text{HpUreE}\) dimer and two equivalents of unlabelled HpUreG monomer differs from that of HpUreE in the absence of HpUreG (Supplementary Figure S6A at http://www.BiochemJ.org/bj/422/bj20091add.htm). A general broadening of the peaks is observed, and a number of them show small chemical shift changes (\(\Delta \delta_{c} \leq 0.1 \text{ p.p.m.}\)), consistent with the formation of a complex between the two proteins, as observed by ITC. The number of glycine resonances does not increase upon complex formation, suggesting maintenance of the UreE homodimeric symmetry. Addition of one equivalent of unlabelled HpUreE dimer to a solution of \(^{15}\text{N}-\text{HpUreG}\) causes broadening beyond detection for most of the backbone amide signals observed in a regular \(^{1}\text{H–}^{15}\text{N}\) HSQC spectrum (results not shown) as compared with the same spectrum of HpUreG [16]. Resonances could be recovered by recording a \(^{1}\text{H–}^{15}\text{N}\) HSQC spectrum (Supplementary Figure S6B), an effect that can be explained with the large molecular mass (approx. 80 kDa) of the HpUreE–HpUreG complex. Small chemical shift changes on selected resonances of backbone amides are observed (\(\Delta \delta_{c} \leq 0.05 \text{ p.p.m.}\)) upon complex formation. An assignment of the resonances, beyond the scope of the present study, would provide information on the surface contact areas, and is currently underway in our laboratories.

The observed 1:2 stoichiometry of the HpUreE–HpUreG complex, coupled to the previously reported dimerization of HpUreE selectively induced by the binding of one equivalent of Zn\text{2+} per protein dimer, and not by Ni\text{2+}–binding [16], prompted us to explore the role of these two metal ions in the stabilization of the protein complex. The SEC elution profile and molar masses of a mixture of HpUreE and HpUreG in a 1:2 ratio, as measured by MALD, were not affected by the presence of Ni\text{2+}, indicating that this metal ion is not capable of significantly stabilizing the protein complex. This is consistent with the absence of a role.
proteins completely disappeared, and a new unique peak, with
M = 79.4 ± 2 kDa and \( K_d = 5.8 ± 0.1 \text{ nm} \), was concomitantly
observed (Figure 4A). This result indicates that the interaction
between \( \text{HpUreE} \) and \( \text{HpUreG} \), leading to the establishment of a
complex, is specifically stabilized by \( \text{Zn}^{2+} \). On the basis of
the theoretical masses of the \( \text{HpUreE} \) dimer (39 kDa) and of the
\( \text{HpUreG} \) monomer (22 kDa), the mass of the new species
formed in the presence of \( \text{Zn}^{2+} \) is fully consistent with the 1:2
stoichiometry established by ITC.

**Molecular model of the \( \text{HpUreE–HpUreG} \) complex**

The viability of the \( \text{HpUreE–HpUreG} \) complex formation was
investigated from a structural modelling point of view.
The model structure of the \( \text{HpUreE} \) dimer was docked on to
the model of the dimeric form of \( \text{HpUreG} \) [16], with optimization
of protein backbone and side chains at the interface between the two
homodimers. In the resulting structure (Figures 5A and 5B),
the two proteins face each other along their extended axes, and only
limited modifications of the proteins backbone, restricted both in
extent and in topology distribution, were necessary in order to
optimize the docking procedure (Figure 5A). The central pocket
formed on the \( \text{HpUreE} \) surface around the conserved Cys\(^{46}\) and
His\(^{68}\) residues matches the shape and volume of the protruding
region around the pair of conserved His\(^{102}\) residues on the surface
of \( \text{HpUreE} \). The shallow crevice formed between the central
C-terminal domain and the peripheral N-terminal domain of
\( \text{HpUreE} \) is filled with the bulge found on the surface of
\( \text{HpUreG} \) around the rim of the protein dimerization interface
(Figures 5A and 5B). Overall, a full size, shape, and electric
charge complementarity between the surfaces of the two proteins
is observed (Figures 5C and 5D), with the formation of the
complex resulting in a large total area \([6378 \, \text{Å}^2 (1 \, \text{Å} = 0.1 \text{ nm})]
40.4 \% of \( \text{HpUreE} \) and 36.3 \% of \( \text{HpUreG} \) that is buried by the
two interacting homodimers. The details of the interaction are
given in the Supplementary Online Data.

**Role of \( \text{Zn}^{2+} \) in the stabilization of the \( \text{HpUreE–HpUreG} \) complex**

A recent article has established the key importance of the surface
exposed conserved Cys\(^{46}\) and His\(^{68}\) residues in \( \text{HpUreG} \) for the
binding of \( \text{Zn}^{2+} \) [16]. Moreover, the present study indicates a
role for His\(^{102}\) and His\(^{152}\) in metal ion binding to \( \text{HpUreE} \).
The structure of the \( \text{HpUreE–HpUreG} \) complex features Cys\(^{46}\), His\(^{68}\)
of \( \text{HpUreG} \), and His\(^{102}\) of \( \text{HpUreE} \), in neighbouring positions in
the central region of the complex. This suggests the building up of
a novel metal-binding site at the interface between the two
protein partners (see close-up in Figure 5A). The position of
His\(^{152}\) cannot be predicted by the model because of the absence
of structural data for the terminal disordered region. In order
to experimentally verify the presence of this site, we carried out
calorimetric titrations of \( \text{Zn}^{2+} \) onto a solution containing a
preformed complex obtained in \textit{situ} by mixing the two proteins
with a 1:2 stoichiometry \( \text{HpUreE} \) dimer:\( \text{HpUreG} \) monomer
(Figure 4C and Supplementary Figure S5). The curve obtained
using the wild-type proteins indeed reveals an event of binding,
characterized by \( K_d = 1.5 ± 0.3 \, \text{ nm} \), which is approx. 2–3 orders
of magnitude tighter than those observed for isolated \( \text{HpUreE} \)
(the present study) or \( \text{HpUreG} \) [16]. This event is distinct from
an additional following binding step with \( K_d = 0.67 ± 0.05 \, \text{ nm} \).
Therefore, the \( \text{HpUreE–HpUreG} \) complex binds two \( \text{Zn}^{2+} \) ions,
in a high-affinity and a low-affinity site.

The identity of the residues involved in these two binding events
was investigated by repeating the same experiment using, instead
of the wild-type proteins, the mutants H102K \( \text{HpUreE} \), H152A
\( \text{HpUreE} \) or C66A/H68A \( \text{HpUreG} \) (Figure 4C) [16]. In the case

![Figure 4](http://www.BiochemJ.org/bj/422/bj4220093add.htm)
of each of the HpUreE mutants, the tight binding event is not observed, whereas it is maintained when the mutant of HpUreG is used. On the other hand, the low-affinity site is still present when the two mutants of HpUreE are utilized, but is disrupted in the case of C66A/H68A HpUreG. The residual binding of two Zn\(^{2+}\) ions in the latter case reproduces what was previously observed for the HpUreG double mutant alone [16]. Overall, these results suggest that His\(^{152}\) and His\(^{152}\) contribute to the building of the high-affinity site in the complex, whereas HpUreG Cys\(^{68}\) and His\(^{68}\) residues are responsible for the low-affinity binding event. The exact topology of these two metal-binding sites cannot be determined at the present stage of the study given the limited structural information on the flexible C-terminal pendant arms containing His\(^{152}\). However, the calculated model structure of the protein complex allows us to speculate that these protein regions could adapt their conformation to fill the cleft that is formed between the proteins’ interaction surfaces (Figure 5B), bringing His\(^{152}\) close to the metal-binding site that also involves His\(^{152}\). On the other hand, the lower affinity binding event could involve a nearby site situated close to Cys\(^{68}\) and His\(^{68}\) on HpUreG. It is worth mentioning here that crystallographic evidence indicates the presence of a dinuclear Zn\(^{2+}\) binding site on the surface of MjHypB (a close homologue to HpUreG), which involves a cysteine and a histidine residue corresponding to Cys\(^{68}\) and His\(^{68}\) on HpUreG [31].

The stabilization of a HpUreE-HpUreG complex in the presence of Zn\(^{2+}\) is significant within the framework of the known role of HpUreG in vivo: this protein is an enzyme that catalyses GTP hydrolysis necessary to the urease activation process [10]. HpUreG belongs to a class of homo-dimeric
GTPases (or ATPases) that use GTP (or ATP) hydrolysis as a conserved molecular switch to regulate a large number of cellular processes [44]. The activity of these hydrolases is, in general, tightly controlled by different factors, such as protein dimerization and subsequent interaction with GAP (GTPase activator protein) and GEF (guanine-nucleotide-exchange factor) proteins. These GTPase regulators are stable functional dimers, as observed for UreE. In order to test a possible role for HprUreE in modulating the enzymatic cycle of HpUreG, we measured the GTPase activity of the HprUreE–HpUreG complex formed in the presence of Zn2+ using both a colorimetric and an enzymatic method, as previously described [16]. We found that the interaction with HprUreE stabilized by Zn2+ is not sufficient to promote any detectable GTPase activity. This result is not surprising: it is known that the GTP-dependent process of nickel incorporation into apo-urease occurs only in the presence of a UreDFG complex, implying that UreD and UreF must also play an essential role in UreG activation.

Conclusions

The results presented here allow us to envisage a mechanism for the urease assembly that entails a specific role for both Ni2+ and Zn2+ ions. An exchange of Zn2+ for Ni2+ binding to HprUreE could be the initial switch that modulates the interaction between HprUreE and HpUreG. Ni2+ released from HprUreE could be incorporated into the apo-urease active site, concomitantly with the Zn2+-induced HprUreE–HpUreG complex formation and consequent stimulation of GTPase activity catalysed by HpUreG. This step would lead to the carbamylation of the lysine residue in the urease active site, thus finalizing the activation of the enzyme.

The chaperones involved in the maturation of [NiFe]-hydrogenase in Escherichia coli, such as HyaP and HyaB [37,38] and HypB [45], also display a specific Zn2+-binding capability. On the other hand, HyaP from H. pylori, responsible for the activation of both urease and hydrogenase enzymes [46], shows Ni2+-binding capability in vitro [47]. The occurrence of a specific interaction between dimeric HyaP and UreE from H. pylori was proved using cross-linking and immuno-blotting experiments, suggesting that this interaction is functional to mediate Ni2+ transfer from HyaP to UreE in vivo [48]. Specific protein–protein interactions were observed between HyaP and HypB during cross-linking experiments, as well as between HypB and UreG from tandem affinity purification [49]. These results, coupled to the observation of specific UreE–UreG interactions, indicate the possible presence of cross-talk mechanisms in vivo, involving HyaP, HypB, UreG and UreE. It is interesting to note that all these proteins possess Ni2+- and/or Zn2+-binding capability. Therefore, the Zn2+-dependent interaction between HprUreE and HprUreG, as well as the interdependence between Ni2+ and Zn2+, emerging in this study for the H. pylori urease system, suggests a functional role for metal binding to these accessory proteins, modulating the formation of the protein–protein complexes necessary for enzyme maturation. These observations represent a paradigmatic general point to understand the role of Zn2+ in the process of Ni2+ delivery and incorporation in different enzymes.

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AUThor ContributiOn

Matteo Bellucci optimized and carried out the preparation of wild-type and mutated HpUreE, as well as the calorimetric titrations. Barbara Zambelli was responsible for gene cloning, the preliminary setup of the protein purification protocol, as well as collection and analysis of CD spectra and light scattering measurements. Francesco Musiani carried out the bio-modelling calculations. Paola Turano planned and performed the NMR experiments. Stefano Ciurli and all co-authors equally contributed to the design of the experiments, analysis of the data, and preparation of the manuscript.

Helicobacter pylori UreE, metal ions and UreG

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SUPPLEMENTARY ONLINE DATA

Helicobacter pylori UreE, a urease accessory protein: specific Ni²⁺- and Zn²⁺-binding properties and interaction with its cognate UreG

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MATERIALS AND METHODS

Protein heterologous expression and purification

According to the sequence of the urease operon from H. pylori 26695 strain (NCBI code NC_000915) [1], the two 31-bp oligonucleotides CACCCTCATGATCATAGGGCGTTTTA-GTTGC and ACTCGAGCTATTTATTACGACCCTTTAAAATC (Novartis) were used to amplify the HpureE gene by PCR, introducing the BspHI and XhoI recognition sites respectively (underlined), using the genome of H. pylori G27 strain as template. The PCR product was double digested with BspHI and XhoI (New England BioLabs) and purified by electrophoresis on a 1% (w/v) agarose gel (Qiagen II gel extraction kit). The DNA fragment was ligated (T4 DNA ligase, Promega) into the pET15b expression vector (Novagen) previously digested with NcoI (generating compatible ends with BspHI) and XhoI endonucleases. The resulting pET15b::HpureE construct was purified from E. coli XL10-Gold Ultracompetent Cells (Stratagene) using the StrataPrep Plasmid MiniPrep Kit (Stratagene), analysed by restriction analysis and sequencing at both strands. The sequence of the cloned gene, obtained by double strand DNA sequencing, has been deposited in the NCBI protein database (code ABM16833). Based on the T7 expression system [2], large-scale expression of HpUreE was obtained using the E. coli BL21(DE3) strain. Cells were grown at 28°C for 48 h in 2 litres of M9 medium (6 g l⁻¹ of Na₂HPO₄, 3 g l⁻¹ of KH₂PO₄, 0.5 g l⁻¹ of NaCl, 1.25 g l⁻¹ of (NH₄)₂SO₄, 0.246 g l⁻¹ of MgSO₄·7H₂O, 5 g l⁻¹ of glycerol and 0.5 g l⁻¹ of glucose), supplemented with 2 g l⁻¹ of lactose for auto-induction of protein expression [3]. The cellular pellet was re-suspended in 100 ml of 20 mM MOPS, pH 6.5, containing 500 mM NaCl, 2 mM dithiothreitol, 10 mM MgCl₂, and 20 μg ml⁻¹ of DNase I. The cells were disrupted by two passages through a French Pressure cell at 20000 psi (1 psi = 6.9 kPa). Cell debris was separated from the supernatant by centrifugation at 15000 g for 30 min at 4°C. Fractionation of the cellular extract into soluble and insoluble portions showed that the over-produced protein (a polypeptide with apparent molecular mass of 20 kDa, in agreement with the theoretical mass of HpUreE monomer, 19.408 kDa) accumulated in the soluble fraction (Figure S1, lane A). According to the high isoelectric point calculated for the HpUreE sequence, the protein was purified using cation-exchange chromatography followed by two size-exclusion separations. The soluble fraction was dialysed twice (5 kDa molecular mass cut-off) for 3 h at 4°C against 3 litres of 20 mM Mes, pH 6.5, containing 2 mM EDTA, and was applied on to a SP-Sepharose XK 16/10 column (GE Healthcare) pre-equilibrated with the starting buffer until the baseline was stable. The protein was eluted with a linear gradient of 200 ml of 0 to 1 M NaCl. Fractions containing HpUreE were combined, concentrated using 10 kDa molecular mass cut-off Centricon ultra-filtration units (Millipore) and loaded on to a Superdex 75 XK 16/60 column (GE Healthcare) equilibrated with two column volumes of 20 mM Tris/HCl, pH 8.0, containing 150 mM NaCl. The eluted protein was further purified using high-resolution size-exclusion chromatography on Superdex 75 HR 10/30 (GE Healthcare), in the same buffer. This protocol provided 15 mg of pure native HpUreE per litre of culture (Figure S1, lane C).

Generation of the H102K and H152A HpUreE mutants was carried out by in vitro site-directed mutagenesis, using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) and the pET15b::HpureE as template, following the manufacturer’s instructions. The mutagenesis primers were CTATGAAATA-GGAAACCGCAAGGCGGCTTTATACTATGGCG/CGCCATA-GTATAAAGCCGCCTTGCGGTTTCCTATTTCATAG for H102K and CTTAACCTGACATGCCCCGACTGAGCCT-TATTCTTAAGGCGCCTTTAAATTAGGCTACTAGCGCGGC-GATGTCACGGGTAAAG for H152A. The sequences of the mutants were verified by sequencing the genes on both strands. Protein expression and purification of the H102K and H152A mutants were carried out as described for the wild-type protein.

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The nucleotide sequence data reported in this study will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number ABM16833.
The purity of the isolated proteins, as well as their molecular mass under denaturing conditions, was estimated by electrophoresis on NuPAGE Novex Pre-Cast Gel System (Invitrogen), with NuPAGE 4–12% Bis-Tris gels stained using the SimplyBlue Safestain (Invitrogen). The identity of the purified protein, recovered from an SDS/PAGE gel, was demonstrated by tryptic digestion, and the similarity between the expected and the experimental protein sequence was confirmed by ESI Q-TOF tandem MS (EMBL Protein Core Facility, Heidelberg). Five digested fragments were sequenced as DLNPLDFNVDH- the SimplyBlue Safestain (Invitrogen), with NuPAGE 4–12 by electrophoresis on NuPAGE Novex Pre-Cast Gel System molecular mass under denaturing conditions, was estimated in complete agreement with the theoretical sequence of HpUreE.

Five digested fragments were sequenced as DLNPLDFNVDH- the SimplyBlue Safestain (Invitrogen), with NuPAGE 4–12 by electrophoresis on NuPAGE Novex Pre-Cast Gel System molecular mass under denaturing conditions, was estimated in complete agreement with the theoretical sequence of HpUreE. The absence of metal ions bound to the purified proteins was confirmed by ICP-ES (inductively coupled plasma emission spectroscopy), using a procedure previously described in [4]. Protein concentration, always expressed by referring to the HpUreE dimer (38815 Da), was estimated using the theoretical extinction coefficient at 280 nm (ε280 = 11460 M⁻¹·cm⁻¹), calculated according to the amino acid sequence using the ProtParam (http://au.expasy.org/tools/protParam.html) web site. This value was confirmed by quantitative analysis of cysteine and methionine sulfur performed using ICP-ES.

### Table S1 Acquisition parameters for the NMR experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dimension of acquired data</th>
<th>Spectral width</th>
<th>Number of scans</th>
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<td>^15N UreG</td>
<td>1k</td>
<td>180</td>
<td>38</td>
</tr>
<tr>
<td>HSQC</td>
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<td>38</td>
<td>32</td>
</tr>
<tr>
<td>^15N UreG + UreE</td>
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<td>128</td>
<td>38</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td>HSQC</td>
<td></td>
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<td>512</td>
</tr>
<tr>
<td>^15N UreE</td>
<td>1k</td>
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<td>40</td>
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<tr>
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<tr>
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<td>40</td>
<td>32</td>
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</table>

### Table S2 Thermodynamic parameters of ZnSO4 binding to the HpUreE–HpUreG complex and its mutant versions

<table>
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<tr>
<th>Protein complex</th>
<th>High affinity binding event</th>
<th>Low affinity binding event</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_d (μM)</td>
<td>ΔH_f (kcal · mol⁻¹)</td>
</tr>
<tr>
<td>HpUreE–HpUreG</td>
<td>1.5 ± 0.3</td>
<td>-22.4 ± 0.2</td>
</tr>
<tr>
<td>H102K HpUreE–HpUreG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H152A HpUreE–HpUreG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HpUreE–C66A/H68B HpUreG</td>
<td>2.0 ± 0.4</td>
<td>-20.1 ± 0.1</td>
</tr>
</tbody>
</table>

Structural modelling of the HpUreE–HpUreG complex

The alignment of BpUreE and HpUreE [5] was used to calculate, using the MODELLER9v5 software [6], 50 structural models of the HpUreE dimer, imposing the structural identity of the two monomers. The structures of the BpUreE [7] and H144*KaUreE [8] dimers (PDB codes 1EAR and 1GMW respectively) were used as templates. The calculated model does not include the region containing the last 23 residues of HpUreE, due to the lack of suitable templates for the homology modelling. Indeed, both in the case of BpUreE and H144*KaUreE, this region was not observed in the crystal structures because of conformational disorder. The best model was selected on the basis of the lowest value of the MODELLER objective function. The results of the PROCHECK analysis [9] for the final model were fully satisfactory. The ROSETTADOCK software [10] was used to calculate an initial complex between the model structure of dimeric HpUreG [11], and the central C-terminal domains of dimeric HpUreE. A search of 1000 complexes was carried out by randomly translating and rotating the initial positions of the interacting proteins. The complex with the best ROSETTADOCK score was selected among all generated models for the subsequent refining run, carried out by applying 1000 times a perturbation to the starting structure. The Cα trace of this complex was used, together with the crystal structures of MjHybP (PDB code 1HF8) [12], BpUreE (PDB code 1EAR) [7], and KaUreE (PDB code 1GMW) [8] as templates to build a model of the HpUreE–HpUreG complex using the MODELLER9v5 software [6]. The alignment included a combination of the sequences of HpUreE with BpUreE [5], and of HpUreE with MjHybP [11]. The calculation, carried out by imposing a structural identity of the two monomers of UreE and UreG, produced 200 structural models. The best model was selected on the basis of the lowest value of the MODELLER objective function. The results of the PROCHECK analysis [9] for the final model were fully satisfactory. The molecular (solvent-excluded) surfaces of dimeric HpUreE and HpUreG were calculated using the UCSF CHIMERA package [13]. All histidine residues were considered neutral. The electrostatic colour-coding was generated using the DELPHI software [14]. This program solves the linearized Poisson-Boltzmann equation to obtain the electrostatic potential in and around the protein, while taking the presence of solvent into account as a high dielectric continuum. The protein internal dielectric constant was set to 4 in all calculations, and the solvent dielectric constant was 80. The salt concentration was set to 0.15 M NaCl, which corresponds to the physiological ionic strength. Most of the atomic contacts between the two proteins (defined as involving atoms positioned at distances < 0.4 Å smaller than the sum of their van der Waals radii) involve hydrophobic interactions between the two polypeptides.
These contacts are localized between the helix that separates the two parallel β-sheets in the N-terminal domain of HpUreE (residues 27–36) and the long central helix of HpUreG (residues 76–88, Figure 5). These interactions are favoured by electrostatic forces between a positively charged patch located on the N-terminal domains of HpUreE (residues 101, 102, 132–137 and 143–149) and a negatively charged region of the HpUreG surface (residues 56, 57 and 75–89). Finally, H-bonds are formed between the side chains of HpUreE Arg\(^{11}\) and HpUreG Ser\(^{38}\), and between the carboxylic group of Glu\(^{83}\) on the HpUreG side and backbone N and O atoms of Lys\(^{32}\), Lys\(^{33}\), Ile\(^{34}\) and Ala\(^{35}\) on the HpUreE side.

Figure S2  ITC data of NiSO\(_4\) and ZnSO\(_4\) binding to wild-type HpUreE and its H152A mutant

Representative plots of raw titration data showing the thermal effect of 30 × 10 \(\mu\)l injections of Ni\(^{2+}\) (100 \(\mu\)M) (A) and Zn\(^{2+}\) (100 \(\mu\)M) (B), on to a wild type HpUreE solution (10 \(\mu\)M). In (C) and (D), the raw data obtained using the H152A HpUreE mutant, under the same experimental conditions as (A) and (B), are shown.

Figure S3 Superimposition of the ¹H-¹⁵N TROSY–HSQC NMR spectra for differently metallated forms of HpUreE

The black trace is that of the apo-protein; the green trace is that of the Ni\(^{2+}\)-bound form; the red trace is that of the Zn\(^{2+}\)-bound protein. A few but significant differences are apparent between Ni-HpUreE and Zn-HpUreE, suggesting different binding modes. All the spectra have been acquired at 800 MHz and 298 K.

Figure S4  Plot of the molar mass distribution for H152A HpUreE in the presence of Ni\(^{2+}\) (red) or Zn\(^{2+}\) (blue)

The solid lines indicate the Superdex S-200 size-exclusion elution profile monitored by the refractive index detector, and the dots are the weight-averaged molecular masses for each slice, measured every second. The average molecular mass and the hydrodynamic radius for the two samples are indicated.
Figure S5  ITC data of HpUreE binding to HpUreG

(A) Raw ITC data showing the thermal effect of 30 × 10 μl injections of 160 μM HpUreE dimer binding to 50 μM HpUreG monomer. (B) ITC data of ZnSO₄ binding to the HpUreE–HpUreG complex. Raw ITC data showing the thermal effect of 30 × 10 μl injections of Zn²⁺ (70 μM) onto a solution containing 5 μM HpUreE dimer and 10 μM HpUreG monomer.

Figure S6  NMR spectral changes induced by the interaction between HpUreE and HpUreG

(A) ¹H-¹⁵N TROSY–HSQC spectrum of the ¹⁵N-HpUreE dimer (black) superimposed with the ¹H-¹⁵N TROSY–HSQC spectrum (red) recorded after addition of unlabelled HpUreG. (B) ¹H-¹⁵N HSQC spectrum of ¹⁵N-HpUreG (black) superimposed with the ¹H-¹⁵N TROSY–HSQC spectrum (red) recorded after addition of unlabelled HpUreE. Solutions of the two proteins were prepared mixing one equivalent of HpUreE dimer with two equivalents of HpUreG monomer. All spectra were acquired at 800 MHz and 298 K.
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