Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

The Ni^{2+} binding properties of *Helicobacter pylori* NikR

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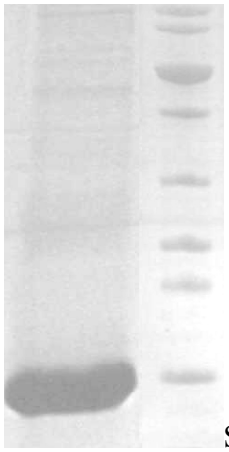
*Hp*NikR expression and purification.

The gene codifying for *Hp*NikR strain G27 (accession number EF127987) was recovered by digesting the previously described *p*ET*15b-nikR* construct[1] with the *Nde*I and *Bam*HI restriction enzymes (New England BioLabs). The isolated fragment, purified by electrophoresis on a 1% (w/v) agarose gel (Qiaex II gel extraction kit), was subsequently ligated (T4 DNA ligase, Promega) into the *p*ET*22b* expression vector (Novagen), digested with the same pair of restriction enzymes. The wild-type sequence of the cloned *Hp*NikR gene was confirmed by double strand DNA sequencing

Based on the T7 system[2], large scale expression of wild-type *Hp*NikR was achieved in 2 L batches of minimum M9 liquid media (1 L contained 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1.25 g of $(NH_4)_2SO_4$, 0.246 g of $MgSO_4$) supplemented with 4 g of glucose per liter of culture. Transformed *E. coli* BL21(DE3) cells, harboring the *p*ET*15b-nikR* construct, were grown at 28 °C and expression was induced, when OD_{600} reached 0.5, by addition of IPTG (isopropyl β-thiogalactopyranoside) to a final concentration of 0.5 mM. The cells were harvested 4 hours after induction by centrifugation at 4,000 *g* for 15 min, at 4 °C. The cells were re-suspended in 40 mL of 50 mM TrisHCl buffer, pH 8 containing 0.5 M NaCl, 2 mM EDTA, and 20 μg mL^{-1} DNAse I, then they were disrupted by two passages through a French Pressure cell (SLM-Aminco) at 20,000 psi. The soluble fraction, obtained after removal of the precipitated material by centrifugation (15,000 *g*, 30 min), was dialyzed against 50 mM TrisHCl buffer, pH 8 containing 2 mM EDTA, and loaded onto a Q-Sepharose XK 26/10 column (GE-Healthcare) that had been pre-equilibrated with two volumes of the

same buffer. The column was washed with the starting buffer until the baseline was stable. The protein was eluted from the column with a 400 mL linear gradient of NaCl (0 to 1 M). The fractions containing *Hp*NikR were collected, EDTA was added up to 10 mM, and the protein was concentrated using 5 kDa MWCO Amicon and Centricon ultra-filtration units (Millipore). The sample was loaded onto a Superdex 75 XK 16/60 column (GE-Healthcare), conditioned with 20 mM TrisHCl buffer, pH 8 containing 0.15 M NaCl and 10 mM EDTA, and 2 mM DTT was added to the eluted protein. The solution was extensively dialyzed against 20 mM HEPES, pH 8 to remove EDTA and DTT, and subsequently concentrated and stored at –80 °C. Prior to each metal analysis and ITC measurement, aliquots of the protein were passed through a Superdex 75 HR 10/30 column (GE-Healthcare) using HEPES as eluting solvent, with the pH chosen for each measurement (vide infra).



SDS-PAGE of purified *Hp*NikR

Protein purity, as well as the molecular mass of *Hp*NikR in denaturing conditions, was estimated by SDS-PAGE according to the method of Laemmli [3], by using a BioRad Mini-Protean II apparatus. Proteins were separated on 15% (w/v) acrylamide-bisacrylamide separating gels, stained using Coomassie brilliant blue R-250. The purified protein, isolated from a denaturing SDS-PAGE gel, was identified by tryptic digestion and ESI Q-TOF mass spectrometry, performed at the EMBL Proteomic Core Facility (Heidelberg). The protein concentration was determined under denaturing conditions (6 M GuHCl), using the theoretical value 8,605 M^{-1} cm^{-1}, calculated from the protein sequence using the ProtParam web site (http://au.expasy.org/tools/protparam.html).

During protein preparation, care was taken to avoid metal contamination. Prior to binding studies, the purified *Hp*NikR was checked for the presence of any metal bound, using inductively coupled plasma emission spectroscopy (ICP-ES), with a procedure previously described.[4] EDTA omission during purification provided a protein sample containing 0.25 equivalents of Ni, 1.2 equivalents of Cu, and 1.2 equivalents of Zn per protein tetramer. The observation of the presence of metals different from Ni in a purified NikR is interesting, because in the published literature on NikR only the nickel content appears to be determined prior to metal binding studies, but is consistent with previous reports indicating that Cu^{2+} and Zn^{2+} strongly bind to the HA sites of NikR.[5] At this stage, incubation of the protein with 50 mM EDTA for 24 hours, followed by extensive dialysis, was able to remove zinc but failed to remove copper (1.0 equivalents still bound), and left 0.1 equivalents of nickel. This observation agrees with previous remarks, reporting a particular resilience of NikR to release nickel in the presence of EDTA.[6, 7] The presence of EDTA in every step of protein purification was necessary in order to yield a fully apo-protein, with metal content below the ICP-ES detection limit. EDTA was then fully removed before each ITC measurements using extensive dialysis followed by size exclusion chromatography.

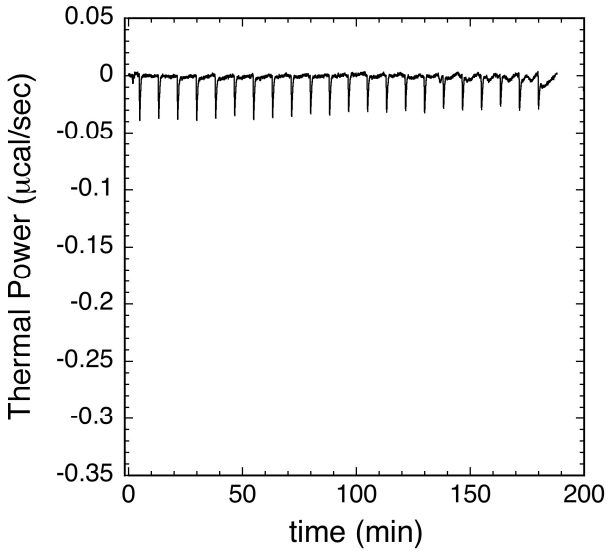
Calorimetric measurements

During protein manipulation, the ionic strength of the protein solution was found to influence the precipitation propensity of *Hp*NikR in the presence of nickel. Protein precipitation was observed after the addition of 20 equivalents of nickel (protein concentration was 40 μM), when *Hp*NikR was diluted in 20 mM TrisHCl pH 8, 150 mM NaCl. This effect was not noticed when NaCl was omitted from the protein solution. In a recent study, *Hp*NikR precipitation in the presence of 12 equivalents of nickel was reported [7]. In the present study, nickel was titrated in a protein sample diluted in 20 mM

HEPES. In these conditions, no protein precipitation was observed after the addition of up to 25 equivalents of nickel per protein tetramer.

Titration experiments were performed at 25 °C using a high-sensitivity VP-ITC microcalorimeter (MicroCal LLC, Northampton, MA). The raw data correspond to the power required by the calorimeter to maintain a constant temperature during and following injection. Each heat pulse corresponds to injection of ligand into the protein solution (Figure 1A of the manuscript). Separate titrations were conducted for the two binding events visible from the raw data, using different starting protein and metal ion concentrations. In each individual titration, small volumes (5-10 μL) of a solution containing $NiSO_4$ (Carlo Erba Reagents, purity grade 99%) at a concentration of 200 μM (high affinity sites, Figure 1B) or 3 mM (low affinity sites, Figure 1C), diluted in 20 mM HEPES in the pH range 6.5-8, were injected into a solution of *Hp*NikR tetramer (4-9 μM for the high affinity sites, 15 μM for the low affinity sites) in the same buffer (cell volume =1.4093 mL, stirring speed = 290 rpm) using a computer-controlled 310 μL microsyringe. Each experiment was started with a small injection of 2 μL, that was subsequently discarded from the analysis of the integrated data, in order to avoid artifact due to the diffusion through the injection port occurring during the long equilibration period, locally affecting the protein concentration near the syringe needle tip. The metal solution was prepared diluting a concentrated stock solution of nickel (100 mM $NiSO_4$) in the buffer conditions used for the last size exclusion purification step. The pH of each solution was checked before starting the analysis. The reference cell was filled with deionized water. Before each experiment, the protein solution was degassed for 2-5 min, to eliminate air bubbles, using the ThermoVac accessory of the microcalorimeter. Care was taken to start the first addition after baseline stability had been achieved. In order to allow the system to reach the equilibrium, a spacing of 500 sec between each ligand injection was applied. Heat produced by protein dilution was verified to be negligible by injecting buffer without nickel into the protein solution. For each titration, a control experiment was set up, titrating the nickel

solution into the buffer alone, under the same conditions, and this heat of dissolution, illustrated below, was subtracted from the observed reaction heats.



Raw titration data of the control experiment, representing the thermal effect of 10 μL injections of Ni^{2+} onto buffer solution

Calorimetric data analysis

Integrated heat data (Figure 1B and 1C) were fitted using a non-linear least-squares minimization algorithm to a theoretical titration curve, using the MicroCal Origin software. ΔH (reaction enthalpy change in cal mol^{-1}), K_b (binding constant in M^{-1}) and n (number of binding sites), were the fitting parameters. The reaction entropy was calculated using the relationships $\Delta G = -RT\ln K_b$ ($R = 1.9872$ cal mol^{-1} K^{-1}, $T = 298$ K) and $\Delta G = \Delta H - T\Delta S$. The reduced chi-square parameter χ_v^2 ($\chi_v^2 = \chi^2/\square$, where $\square$ is the degrees of freedom, $\square = N_{idp} - N_{par}$, N_{idp} = number of points, and N_{par} = number of parameters floating in the fit)was used to establish the best fit. Several different schemes of binding were utilized to attempt the fits of the data, which involved either one or two sets of sites, as well as sequential binding sites. The best fits were consistently obtained using the two sets of sites scheme for the high affinity sites and the one set of site scheme for the low affinity sites. The results are shown in Table 1SI and the curves associated to each best fit are illustrated below.

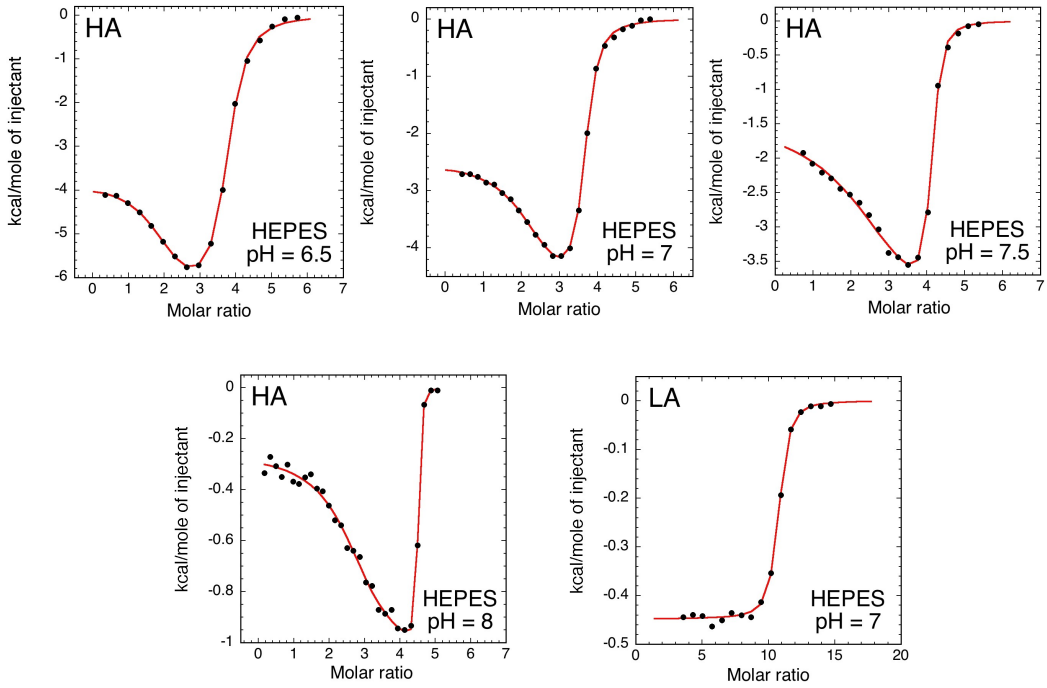


Table 1SI. Results of the fitting analysis for the binding of Ni^{2+} to *Hp*NikR in 20 mM HEPES buffer at 25 °C for the high-affinity (HA) and low-affinity (LA) sites, using different binding schemes

HA sites	pH	Binding scheme	n	K_b (M^{-1})	K_d (nM)	ΔH^a	ΔS^b	χ_v^2
	6.5	Two sets of sites	n_1=1.8±0.1	8.2±3.1 x 10^7	12±5	-3.8±0.1	23.3	3373
			n_2=1.8±0.1	6.2±0.7 x 10^6	160±20	-6.8±0.3	8.3	
	6.5	Sequential binding sites	n= 4	1.1±0.6 x 10^7	91±49	-5.3±0.5	14.5	9286
				1.1±0.7 x 10^7	91±58	1.7±1.9	37.9	
				1.3±0.6 x 10^7	77±36	-1.4±0.2	-13.8	
				1.6±0.3 x 10^6	625±118	-2.1±0.5	21.4	
	7.0	Two sets of sites	n_1=2.2±0.1	8.5±1.8 x 10^7	12±3	-3.8±0.1	27.9	1359
			n_2=1.4±0.1	8.0±0.5 x 10^6	125±8	-6.8±0.3	14.2	
	7.0	Sequential binding sites	n= 4	1.2±0.6 x 10^6	833±415	-19±6	-35.8	6524
				2.9±0.9 x 10^7	34±11	23±3	112	
				1.3±0.3 x 10^7	77±18	-17±2	-25.2	
				1.3±0.2 x 10^6	770±118	0.03±0.3	28	
	7.5	Two sets of sites	n_1=2.4±0.2	1.3±0.7 x 10^8	8±4	-3.8±0.1	32.2	4385
			n_2=1.6±0.2	2.5±0.6 x 10^7	40±10	-6.8±0.3	17.6	
	7.5	Sequential binding sites		2.1±0.2 x 10^6	91±49	-22±16	-46.9	12940
				1.2±0.8 x 10^8	91±58	35±20	155	
				8.7±4.2 x 10^7	77±36	-22±8	-39.6	
				1.1±0.3 x 10^7	630±118	-0.1±0.4	31.9	
	8.0	Two sets of sites	n_1=2.6±0.1	2.1±1.3 x 10^9	0.5±0.3	-3.8±0.1	41.8	686.8
			n_2=1.8±0.1	1.7±1.0 x 10^8	6±4	-6.8±0.3	33.9	
	8.0	Sequential binding sites		1.5±2.0 x 10^5	6.7±8.9 x10^3	-3.9±5.9	10.6	7344
				9.0±12 x 10^6	111±148	4.4±7.1	46.7	
				2.6±3.0 x 10^5	3.8±4.4 x10^3	12±15	64.4	
				2.6±2.8 x 10^7	38±41	-15±15	-16.9	
LA sites	**pH**	**Binding scheme**	**n**	**K_b (M^{-1})**	**K_d (□M)**	**ΔH^a**	**ΔS^b**	**χ_v^2**
	7.0	One set of sites	n=10.4±0.1	2.1±0.4 x 10^6	0.5±0.1	-0.5±0.1	27.2	205

[a] kcal mol^{-1}, [b] cal mol^{-1} K^{-1}

Circular dichroism spectroscopy

The CD spectra of apo-*Hp*NikR and Ni_4-*Hp*NikR (0.26 mg mL^{-1}) in 20 mM phosphate buffer, pH 7.6, was recorded at 25 °C, using a Jasco J-810 spectropolarimeter, flushed with N_2, and a cuvette with 0.1 cm path-length. The spectra were registered from 185 to 250 nm at 1 nm intervals. Ten spectra were accumulated at room temperature, and averaged to achieve an appropriate signal-to-noise ratio. The spectrum of the buffer was subtracted:

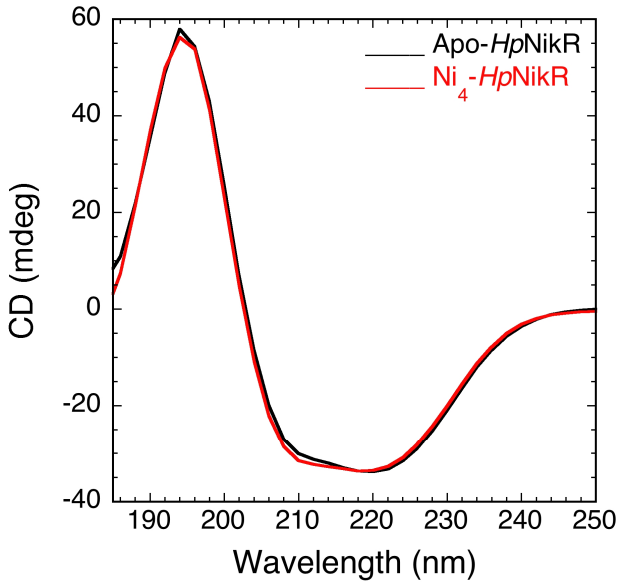


Figure 1ESI. Circular dichroism spectra of *Hp*NikR in the absence (black line) or presence (red line) of four equivalents of $NiSO_4$.

Light scattering measurements

*Hp*NikR (100 µL, 60 µM) in TrisHCl 20 mM (pH 7.6), 150 mM NaCl, was loaded onto a Superdex-75 HR 10/30 column (GE-Healthcare) pre-equilibrated with the same buffer, and eluted at room temperature 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). Quasi-elastic

(dynamic) light scattering data were collected at 90° angle using a WyattQELS device. The concentration of the eluted protein was determined using a refractive index detector (Optilab DSP, Wyatt). Values of 0.180 for the refractive index increment (dn/dc) and 1.3219 for the solvent refractive index were used. Molecular weights were determined from a Zimm plot. Data were analyzed using the Astra 5.1.7 software (Wyatt Technology), following the manufacturer's indications.

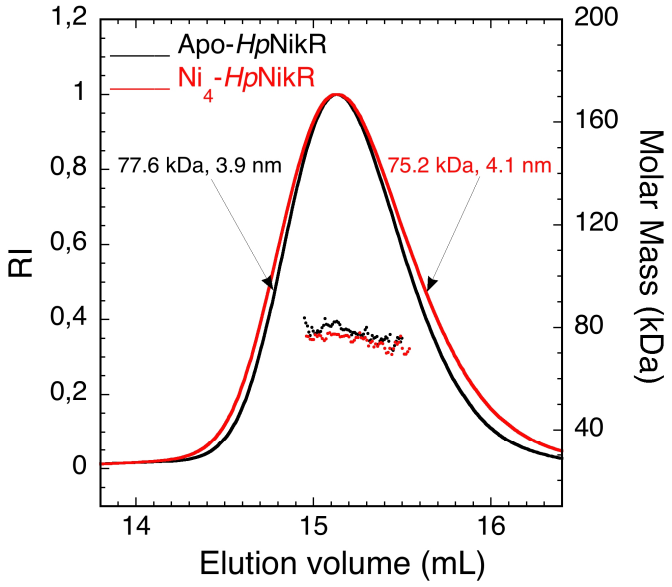


Figure 2ESI. Molar mass distribution plot for *Hp*NikR as established by on-line size-exclusion chromatography, multiple angle light scattering (MALS), and quasi-elastic light scattering (QELS). The black and red lines indicate the traces from the refractive index detector for HpNikR in the absence and presence of four equivalents of $NiSO_4$, respectively. The dots are the weight-average molecular weights for each slice, measured every second. Estimates of the hydrodynamic radii for the apo- and holo-protein as determined by quasi-elastic light scattering (QELS) and indicated in the figure, prove that nickel binding does not induce changes in the protein hydrodynamic behavior, suggesting that the conformational rearrangement allowing the protein to bind DNA does not significantly affect its overall architecture.

Buffer pH-titration

In order to reveal possible binding of Ni^{2+} to HEPES, we determined, using pH potentiometric titrations the pK_a value of the a 20 mM buffer solution in the absence and presence of increasing amounts of metal ion ($NiSO_4$ from Carlo Erba) ranging from 100 μM to 20 mM. The measurements

were performed in a double-walled glass vessel at 25±0.1 °C with a Radiometer Analytical TIM 860 titration manager. Purified nitrogen was bubbled through the solutions during titrations. The HEPES (Sigma) solution was titrated with standard CO_2-free KOH in the pH range 5.0 – 10.0. The electrode was calibrated in both the acidic and alkaline regions by titrating 0.01 M HNO_3 under the same experimental conditions. If an interaction between HEPES and Ni^{2+} occurred, we would expect a shift of the pK_a to lower values, as monitored using dpH/dV plots. In fact, no such a shift was observed, leading us to conclude that HEPES is an innocent buffer for Ni^{2+} and that, under the experimental conditions used for the microcalorimetric measurements of the binding constant between Ni^{2+} and *Hp*NikR, the metal ion is present in solution in its solvated aquo complex.

Buffer NMR Spectroscopy

In order to further confirm the absence of binding of Ni^{2+} to HEPES, we recorded the ^1H NMR spectra of 20 mM HEPES in the absence and presence of increasing amounts of metal ion ($NiSO_4$ from Carlo Erba) ranging from 100 μM to 20 mM, using an Varian INOVA 600 MHz spectrometer. Ni^{2+} is a paramagnetic metal ion (S=1) known to induce large chemical shift changes and/or signal line width broadening onto the coordinating ligands,[8] and the formation of such a complex would be easily revealed by monitoring the proton spectrum of HEPES in different metal ion concentrations. Indeed, we observed no changes in chemical shift, no decrease of intensity, and no significant line broadening up to 1:1 metal to buffer ratio, a strong indication for the absence of a coordination complex between Ni^{2+} and HEPES.

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