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

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The binding constants between Ni^{2+} and *Helicobacter pylori* NikR have been determined using isothermal titration microcalorimetry in order to rationalize the role of this protein as a nickel-dependent biological sensor.

In the human pathogen *Helicobacter pylori*, Ni^{2+} is essential for the activity of urease and hydrogenase, devoted, respectively, to pH-buffering of the acidic gastric mucosa layers,[1,2] and to consumption of nutrient hydrogen.[3] However, Ni^{2+} is also potentially toxic and its cellular content and trafficking must be tightly controlled. *H. pylori* NikR (*Hp*NikR) is a transcription factor that performs this regulatory role by repressing and activating genes that code for Ni^{2+}-enzymes or proteins involved in Ni^{2+} homeostasis.[4–8] The understanding of the mechanism of action of *Hp*NikR as a nickel-sensor and transcriptional regulator requires clear determination of its Ni^{2+} binding properties.

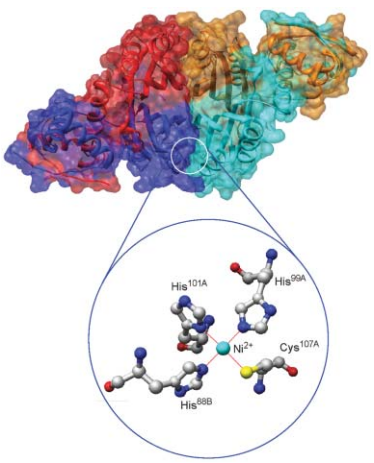
The structural architecture of *Hp*NikR[9] (Scheme 1) is similar to the structures of its orthologs from *E. coli* (*Ec*NikR)[10,11] and *Pyrococcus horikoshii* (*Ph*NikR).[12] The protein is a homotetramer made of a dimer of dimers, with each monomer composed of two distinct regions: an internal C-terminal tetramerization or metal-binding (MBD) domain, and an external N-terminal DNA-binding domain (DBD) containing a ribbon–helix–helix motif found in several transcription factors.

The structures of the Ni-bound forms of *Ec*NikR and *Ph*NikR reveal a set of four identical so-called high affinity (HA) sites at the inner interface between the two pairs of MBD domains. Each site involves four fully conserved residues (two His and one Cys from one monomer and one His from the adjacent monomer) to yield a square-planar Ni^{2+} coordination (Scheme 1).[11,12] The same 4 : 1 stoichiometry was determined for *Hp*NikR.[13,14]

We have carried out a study of the Ni^{2+}-binding thermodynamic properties of *Hp*NikR at 25 °C using isothermal titration calorimetry (ITC), in HEPES. This buffer is ideal for metal binding studies because it does not form complexes with a large series of divalent metal ions,[15–17] as also confirmed in this study for the case of Ni^{2+} using pH-potentiometric measurements and ^{1}H-NMR spectroscopy (see Supporting Information, ESI†). This warrants that Ni^{2+} is present in solution, under the indicated experimental conditions, as the aquo ion and not in a complex with the buffer, so that the measured binding constants refer to the actual reaction of the solvated ion with the protein, and not to the exchange reaction between a Ni^{2+}-buffer complex and the protein itself.

In the case of previous reports on *Ec*NikR and *Hp*NikR, the dissociation constants for Ni^{2+} binding to the HA sites were estimated by monitoring the electronic absorption band at 302 nm as a function of Ni^{2+} added in the presence of the chelating competitor EGTA.[13,18,19] It is known that the presence of EGTA inhibits NikR binding to DNA,[18] thus implying that this exogenous ligand might be present in a ternary complex with the protein and Ni^{2+}. This would affect the estimate of the dissociation constant of the Ni^{2+}–NikR complex. In addition, the correct analysis of the competition experiment implies the knowledge of the dissociation constant of the EGTA–Ni^{2+} complex, while in fact, there is considerable disagreement on this value in the literature.[20] Moreover, it is known that EGTA forms polynuclear complexes with Ni^{2+}, with dissociation constants varying by several orders of magnitude,[21] adding even more uncertainty to the values to use in order to correctly interpret the reported competition experiments. In the present case, a clean titration method was applied, with no additional metal chelator present in solution except for the protein itself.

In a typical ITC titration experiment (Fig. 1A), integration of the peaks obtained upon Ni^{2+} binding to fully apo-*Hp*NikR yields the enthalpy change that follows each injection (Fig. 1B,C). The data indicate the presence of a tight binding event, finished after four equivalents of Ni^{2+} added per *Hp*NikR tetramer. This



Scheme 1 Ribbon/surface scheme of the tetramer of *Hp*NikR (PDB code 2CAD†) showing the details of the high-affinity (HA) nickel-binding site.

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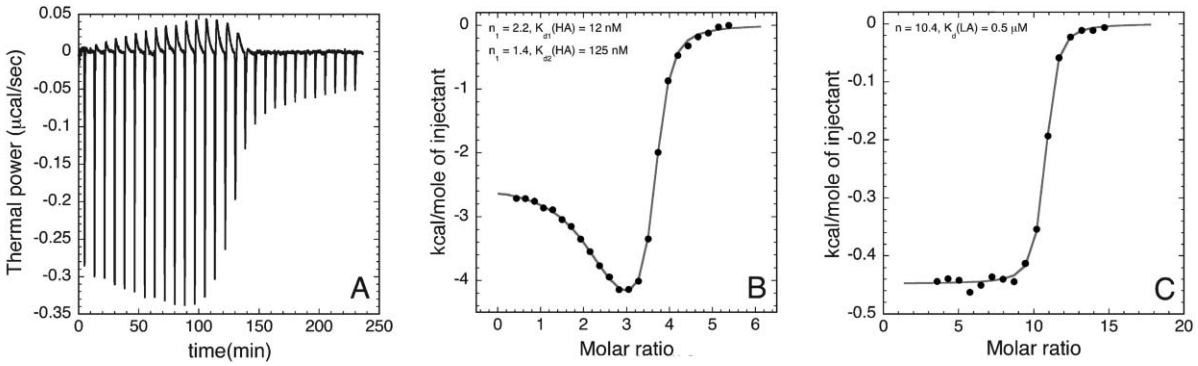

Fig. 1 ITC titration data for the binding of $NiSO_4$ to *Hp*NikR at 25 °C in 20 mM HEPES buffer, pH 7.0. (**A**) Raw titration data represent the thermal effect of 28 × 10 μL injections of Ni^{2+} (200 μM) onto protein solution (7 μM). Normalized heat of reaction data for the high affinity (**B**) and low affinity (**C**) binding events were obtained integrating the raw data, and subtracting the heat of ligand dilution into the buffer. For (**C**), the protein concentration was 15 μM and the Ni^{2+} solution was 3 mM. The solid line represents the best fit of the integrated data, obtained by a non-linear least squares procedure. The calculated number of sites and dissociation constants are indicated.

observation is consistent with the metal-binding stoichiometry previously observed for *Ec*NikR, *Ph*NikR, and *Hp*NikR.

Attempts to fit the integrated heat data (Fig. 1B) were carried out using several different binding schemes (Table 1 ESI†). Best fits could be obtained using a model involving two different types of binding sites, each presenting a stoichiometry of *ca.* two ions, suggesting that in solution the overall four HA Ni^{2+}binding sites are grouped in two distinct pairs (Table 1). Considering the identity of the residues building each Ni^{2+} HA site, the observation of two sets of different high-affinity sites, with binding constants differing by an order of magnitude, could stem from the different orientation of the N-terminal domains relative to the C-terminal oligomerization domains, as seen in several X-ray structures of NikR proteins from different biological sources. This observation is also consistent with the presence of two, and not four, Ni^{2+} ions bound in the structure of *Hp*NikR, obtained by soaking the crystal of apo-protein with $NiSO_4$ at low pH = 4.6, in conditions that would produce a non-optimal binding.[9]

The dissociation constants resulting for the two types of HA sites at pH 7.0 are K_{d1}(HA) = 12 ± 3 nM and K_{d2}(HA) = 125 ± 8 nM. These values are *ca.* 4–5 orders of magnitude higher than those previously reported for *Ec*NikR (K_d = 1–7 pM)[18,19] and *Hp*NikR (K_d = 4 pM),[13] a difference possibly ascribed to the different analytical techniques and experimental conditions utilized for the measurement.

The affinity of *Hp*NikR for Ni^{2+} shows a strong dependence on pH in the range 6.5–8, with binding constants increasing with increasing pH (Table 1). This observation indicates a proton dissociation event linked to Ni^{2+} binding. The value of the pK_a for the group involved in this process cannot be estimated because the binding constant extends beyond the ITC detection limit (*ca.* 10^9) at pH ⩾ 8. However, the observed trend of the binding constants is consistent with the cysteines in the high affinity sites being the residues responsible for the proton dissociation event occurring upon Ni^{2+} binding. The two tight binding processes are both enthalpically ($\Delta H < 0$) and entropically ($\Delta S > 0$) driven (Table 1). The values given for ΔH and ΔS are apparent, and include contributions not only from Ni^{2+} binding but also from associated events such as deprotonation of the cysteines and consequent change in the buffer ionization state.

While the four HA sites are being filled, a second event, endothermic in nature, follows the fast initial exothermic metal binding step (Fig. 1A). This process evolves with a mono-exponential decay indicative of a slow first order process (k = (8 ± 3) × 10^{-3} s^{-1}). Spontaneous endothermic processes must be driven by positive entropy, usually involving release of water molecules from the hydration sphere of the protein, due to either conformational changes and/or protein oligomerization. The latter possibility was excluded using multiple angle light scattering measurements (MALS, Fig. 1 ESI†), which demonstrated that the

Table 1 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

HA sites	pH	n	K_b (M^{-1})	K_d (nM)	ΔH^a	ΔS^b
	6.5	n_1 = 1.8 ± 0.1	8.2 ± 3.1 × 10^7	12 ± 5	−3.8 ± 0.1	23.3
		n_2 = 1.8 ± 0.1	6.2 ± 0.7 × 10^6	160 ± 20	−6.8 ± 0.3	8.3
	7.0	n_1 = 2.2 ± 0.1	8.5 ± 1.8 × 10^7	12 ± 3	−3.8 ± 0.1	27.9
		n_2 = 1.4 ± 0.1	8.0 ± 0.5 × 10^6	125 ± 8	−6.8 ± 0.3	14.2
	7.5	n_1 = 2.4 ± 0.2	1.3 ± 0.7 × 10^8	8 ± 4	−3.8 ± 0.1	32.2
		n_2 = 1.6 ± 0.2	2.5 ± 0.6 × 10^7	40 ± 10	−6.8 ± 0.3	17.6
	8.0	n_1 = 2.6 ± 0.1	2.1 ± 1.3 × 10^9	0.5 ± 0.3	−3.8 ± 0.1	41.8
		n_2 = 1.8 ± 0.1	1.7 ± 1.0 × 10^8	6 ± 4	−6.8 ± 0.3	33.9
LA sites	pH	n	K_b (M^{-1})	K_d (μM)	ΔH^a	ΔS^b
	7.0	n = 10.4 ± 0.1	2.1 ± 0.4 × 10^6	0.5 ± 0.1	−0.5 ± 0.1	27.2

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

protein remains a tetramer independently of the presence or absence of four equivalents of Ni^{2+}. On the other hand, a slight modification of the circular dichroism spectrum was observed upon binding of four Ni^{2+} equivalents (Fig. 2 ESI†). Structural differences have been observed between the apo- and holo-forms of *Ec*NikR by circular dichroism,[18] as well as by comparing the crystal structures of apo- and holo-*Ec*NikR and *Ph*NikR orthologues, in which a short helical fragment is formed upon Ni^{2+}-binding to the HA sites.[10,12] In *Ec*NikR, an allosteric mechanism specifically induced by Ni^{2+} binding to the HA sites has been proposed to be responsible for the increased affinity of the protein to DNA.[23] Therefore, it is possible that the slow process occurring as a consequence of Ni^{2+} binding to the HA sites of *Hp*NikR is due to a small change in the protein fold, involving either or both secondary and tertiary structures. This is suggested also by the increasing amplitude of this process as the four HA sites are being filled, and by its disappearance upon addition of four Ni^{2+} equivalents. This folding rearrangement could be responsible for the observed binding of NikR to DNA only when these sites are filled.[13,18,22] Such Ni^{2+}-induced conformational rearrangement, coupled to the observation of two different pairs of HA sites presenting diverse affinities for Ni^{2+}, suggests that the binding of four high-affinity Ni^{2+} ions occurs in a discrete fashion, the first two Ni^{2+} ions binding in a 2 : 1 complex and inducing a preliminary protein conformational change, possibly already able to bind DNA. This process is subsequently completed by the interaction with further two Ni^{2+} ions, providing the final 4 : 1 stoichiometry.

It is known that the affinity of NikR to DNA increases in the presence of Ni^{2+} in excess to the stoichiometry required to fill the HA sites.[13,18,22,24] This observation indicates the presence of additional Ni-binding low affinity (LA) sites. In *Ec*NikR, a dissociation constant of *ca*. 30 μM for the LA sites was estimated.[24] We studied this binding event for *Hp*NikR, using ITC (Fig. 1C).The titration was completed only after addition of *ca*. 15 Ni^{2+} equivalents. A fit of the data to equations corresponding to the one set of sites binding scheme (Table 1) indicates that the protein binds *ca*. 10 additional Ni^{2+} with a dissociation constant K_d(LA) = 0.5 $\pm$ 0.1 μM. The reduced affinity of the LA sites as compared to the HA sites appears to be mainly due to a smaller negative enthalpic contribution. This could be explained with a different type of ligands to Ni^{2+} in the HA sites (N- and S-based His and Cys residues) *vs*. the LA sites. In this regard, it is important to consider that, in the structure of Ni-*Ph*NikR, a set of two symmetric metal-binding sites, in addition to the four HA sites, was identified by crystallography at the interface between the MBD and the DBD regions, and was proposed to lock the relative position of the two domains in a closed/cis conformation.[12] In this type of sites, Ni^{2+} is bound to O-based ligands (Glu32 and Asp36 side chains). In *Ec*NikR, a K^+ ion is bound to the carboxylic O atoms of conserved Glu30 and Asp34 in a binding site corresponding to that observed for *Ph*NikR. In *Ec*NikR, this binding site for K^+ appears to be functional only in the presence of a bound DNA fragment representing the protein operator,[11] as if the metal ion imposed a relative positioning of these protein domains that is optimal for DNA-binding. The presence of O/N donors to Ni^{2+} in the LA sites has also been identified using Ni K-edge X-ray absorption spectroscopy.[23] This low affinity site was not found to bind Ni^{2+} in *Hp*NikR, possibly because of the peculiar soaking conditions.[9]

In conclusion, the present study has established that i) *Hp*NikR binds two Ni^{2+} ions with a K_d of *ca*. 10 nM and two additional Ni^{2+} ions with a K_d of *ca*. 100 nM in four high-affinity sites, in contrast to the previously reported pM dissociation constants obtained using competition experiments; ii) the nickel-affinity of *Hp*NikR in the high affinity sites shows a strong dependence on pH in the 6.5–8.0 range, with binding constants larger than 10^9 above pH 8; iii) a conformational rearrangement with a kinetic constant in the order of 10^{-3} s^{-1} occurs upon Ni^{2+} binding to the high-affinity sites; iv) *Hp*NikR binds excess Ni^{2+} with lower affinity (LA sites), with dissociation constants in the order of μM; v) this lower affinity is due to the enthalpic term, suggesting a different type of ligand type for the low-affinity *vs*. the high-affinity sites. These results shed new light onto the molecular properties of *Hp*NikR by providing a clear link between the structure and the functional role of this biological nickel sensor.

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