Sub-micromolar affinity of *Escherichia coli* NikR for Ni(II)†

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The dissociation constant of Ni(II) for *Escherichia coli* NikR was determined using three independent techniques, including binding kinetics, and shown to be in the sub-micromolar range.

NikR is a Ni(II)-responsive transcription factor that regulates levels of Ni(II) in *Escherichia coli* and other bacteria such as *Helicobacter pylori*.[1] In *E. coli*, NikR is involved in nickel homeostasis through Ni(II)-dependent repression of the nikABCDE operon, thus suppressing expression of the ABC transporter specific for the import of Ni(II). *E. coli* NikR consists of 133 amino acids, of which 12 are histidines, and forms a tetrameric structure with several Ni(II)-binding sites.[2–5] One of these sites has square planar His_3Cys coordination[3,6] and is buried at the interface between the two dimer pairs making up the tetramer.[2] A picomolar dissociation constant has previously been ascribed for this high affinity (HA) site, using competition assays.[7] This report presents a re-evaluation of the Ni(II) binding properties of *E. coli* NikR using direct measurements: UV-vis equilibrium titrations, filter binding assays, and rapid kinetics experiments.

Ni(II) binding to the HA site activates NikR for operator binding (K_D ~30 nM). Ni(II) binds to several other (low affinity, LA) sites, which may number up to 7 per monomer.[8] These are less well characterized, and have a lower affinity (0.03–50 μM).[7] Ni(II) binding to (one of the) LA sites increases the affinity of NikR for DNA about 1000-fold (K_D ~10–20 pM).[7] This implies that NikR is sensitive or responsive to two greatly different Ni(II) concentrations, and thus acts at two distinct levels of regulatory control. This feature of NikR is, to our knowledge, unique for a metallo-regulatory protein. The cellular levels of Ni(II) in living *E. coli* cells have been estimated to lie between 10^{-8} and 10^{-6} M,[9] and the experimentally determined K_D lies in the low μM range for many bacterial Ni(II)-binding proteins.[10] An apparent inconsistency thus exists between the pM affinity of NikR for Ni(II) on the one hand, and cellular nickel levels and affinities of Ni(II) binding proteins on the other hand. This clearly warrants further investigation, which is reported here. We have chosen for direct titration techniques to study Ni(II) binding by *E. coli* NikR, rather than assays based on chelator–protein competition, which may lead to errors in interpretation (*vide infra*). We find that the HA site of NikR certainly does not have pM affinity for Ni(II), but rather an affinity four orders of magnitude lower, consistent with NikR being responsive to sub-micromolar levels of nickel. Similarly, in a recent study on *H. pylori* NikR,[11] the affinity for Ni(II) as determined by a direct assay (isothermal titration calorimetry) was three orders of magnitude lower than previously determined by competition assays suggesting consistent overestimation of NikR binding strength with indirect measurements using EGTA.[12]

NikR‡ binds Ni(II) in the HA site with square planar geometry and with cysteine ligation,[2,6] as observed by UV-vis spectroscopy.[4,8] Increase of the Cys $S^{\gamma} \rightarrow$ Ni LMCT band at 302 nm (Fig. 1a) is linear up to one Ni(II) equivalent, after which mass aggregation is seen, which is complete at less than 1.5 equivalents of Ni(II).[8] Binding of Ni(II) is still possible by the aggregate however, between 4 and 7 Ni(II) ions per NikR monomer.[8] No aggregation at all is observed with 0.1 or less equivalents of Ni(II) using dynamic light scattering (see ESI†).

In general, when the concentration of Ni(II) is much lower than that of NikR, Ni(II) binding is approximately linear. The proportion of bound Ni(II) is expected to decrease as the [NikR] of the experiment is lowered and nears the actual K_D of the binding site. As justified in the ESI,† this leads to the

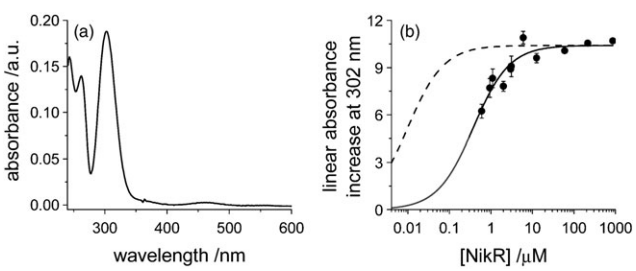

Fig. 1 (a) UV-Vis difference spectrum of 215 μM NikR in the presence and absence of *circa* 20 μM $NiSO_4$. (b) Dependence of the linear absorbance increase at 302 nm of Ni(II)-bound NikR as a function of Q2E NikR concentration (●). The data were fitted (line) to a hyperbolic function (eqn (1)). The plateau value corresponds to the extinction coefficient. For illustrative purposes, the expected dependence for binding with picomolar affinity (K_D = 10 pM) is shown (dashed line).

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† Electronic supplementary information (ESI) available: Additional experiments on the protein aggregation state, experimental details of binding assays and stopped-flow binding kinetics, and details on data fitting procedures and binding models used. See DOI: 10.1039/b719676h

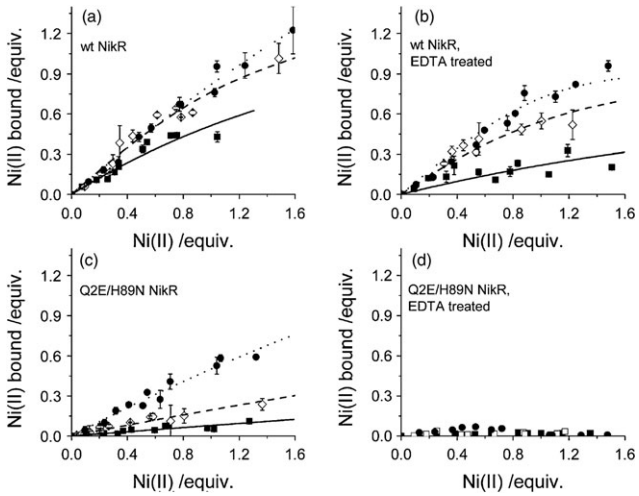

Fig. 2 Ni(II) binding by wt NikR (a), wt NikR washed with EDTA (b), Q2E/H89N NikR (c) and Q2E/H89N NikR washed with EDTA (d), respectively, as determined using a filter binding assay. The concentration of NikR is 0.1 μM (■), 0.2 μM (◇) and 0.4 μM (●), and fits to the data are shown as solid, dashed and dotted lines, respectively.

fraction of the total Ni(II) that is bound to the HA site to show a hyperbolic dependence on [NikR], and to be proportional to the linear increase in absorbance at 302 nm as a function of Ni(II). This relation is expressed in eqn (1):

$$\text{linear absorbance increase} = \varepsilon_{302} \cdot \frac{[\text{NikR}]_{\text{tot}}}{(K_{\text{D}} + [\text{NikR}]_{\text{tot}})} \quad (1)$$

The experimentally determined§ linear absorbance increase due to Ni(II) binding to the HA site indeed shows a hyperbolic dependence on the NikR concentration (Fig. 1b), that can be excellently fitted using eqn (1). The fit yields $K_{\text{D}} = 0.379 \pm 0.068$ μM and $\varepsilon_{302} = 10\,400 \pm 300$ $\text{M}^{-1}\,\text{cm}^{-1}$. Earlier reports are confusing in this aspect, with ε_{302} varying between 3200 and 7200 $\text{M}^{-1}\text{cm}^{-1}$.[4,7a,b] The differences in absorbance intensities are very small at very low NikR concentrations, precluding determination of the linear absorbance increase at [NikR] < 0.6 μM. Nevertheless, the downward tendency of the linear absorbance increase at lower NikR concentration is very clear and it is incompatible with picomolar affinity. At the concentrations applied, NikR remains tetrameric.[8]

Ni(II) binding to NikR was also determined using a filter binding assay.¶ The amount of ^{63}Ni(II) bound as a function of NikR concentration is shown in Fig. 2a and b for wt NikR and EDTA-treated wt NikR, respectively. No difference in Ni(II) binding was observed between Q2E and wt NikR (not shown), indicating that the Q2E mutation does not affect the Ni(II) sites. EDTA-treated NikR contains Ni(II) only in the HA site (as concluded from UV-vis experiments and metal quantification),[8] whereas the non-treated sample also contains Ni(II) bound to other sites.[8] This is because Ni(II) is only very slowly released from the HA site.[8] We also studied the double mutant Q2E/H89N NikR (Fig. 2c and d), which does not bind Ni(II) in the HA site since His89 is missing (indeed, EDTA-washed Q2E/H89N NikR does not retain Ni(II), Fig. 2d). In short, the experiments shown in Fig. 2a to d, respectively, indicate

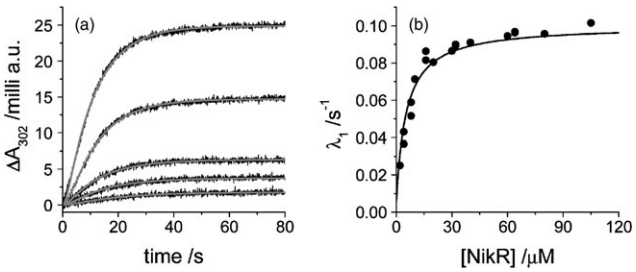

Fig. 3 (a) Stopped-flow traces of Ni(II) binding by NikR. Here, NikR concentration was 2, 4, 8, 16 and 32 μM and $NiSO_4$ was 0.2, 0.4, 0.8, 1.6 and 3.2 μM, respectively. The data (black lines), corrected for background absorbance, were fit globally (grey lines) to a two-step binding mechanism. (b) Exponential rate constants of the slow phase (λ_1) as a function of NikR concentration. The rate constants (●) were derived by fitting the traces to a bi-exponential function. The fast phase (not shown) shows too much scattering to be useful for analysis. The line through the data is a simulation using the kinetic constants obtained by numerical fitting.

binding to the HA and LA sites (panel a), binding to HA only (panel b), binding to the LA sites only (panel c), and background binding (panel d).

The data were fit using Dynafit,[13] assuming independent binding to two categories of binding sites, each with a different affinity for Ni(II). The HA site is considered one category, while all other sites that are rapidly depleted by EDTA are considered the second category (LA). The best fits to all binding data yield average K_{D} values of 82 ± 24 nM for the HA site (78.9 and 85.7 nM for the Q2E and wt proteins, respectively) and 2.2 ± 0.7 μM for the LA sites assuming three additional sites per NikR monomer. Note that if a site of pM affinity were present, a curve with a slope of unity would be observed up to one Ni(II) equivalent, with no relevant difference between the different protein concentrations used.

In a third experimental approach, we determined the kinetics‖ of Ni(II) binding to the HA site (Fig. 3). Fitting of the stopped-flow traces to a mono-exponential function gives imperfect fits (not shown), with deviations at the beginning of the reactions (*i.e.* a lag-phase). The approximate rate constants derived from this fitting display a hyperbolic dependence on NikR concentration. Together, this is evidence of a binding mechanism with a colourless intermediate.[14] A two-step binding mechanism (Scheme 1) is the most simple and from a structural point of view, the most likely mechanism; the HA site is in the interior of the protein, and the most direct route for Ni(II) to this site is through a cluster of His residues located on the surface.[2,5]

The data were fit globally using Dynafit[13] to this mechanism (see ESI for details†), yielding the following values for the kinetic constants: $k_1 = 3.65\ (\pm 0.46) \times 10^4\ \text{M}^{-1}\,\text{s}^{-1}$, $k_{-1} = 0.095 \pm 0.015\ \text{s}^{-1}$, $k_2 = 0.088 \pm 0.016\ \text{s}^{-1}$ and $k_{-2} = 0.0123 \pm$

$$\text{Ni(II)} + \text{NikR} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{Ni(II)}\bullet\text{NikR}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Ni(His}_3\text{Cys)NikR}$$

Scheme 1 Two-step binding mechanism of Ni(II) to the HA site of NikR.

$0.0012\ s^{-1}$ ($k_{on} = 1.76 \times 10^4\ M^{-1}\ s^{-1}$, $k_{off} = 0.0064\ s^{-1}$). The K_D derived from these values is 0.363 (±0.105) μM.

We clearly show here, using three distinct methods, that NikR binds Ni(II) in the HA site with sub-micromolar affinity. In addition to this site, Ni(II) also binds to several other sites, with slightly lower affinity. The affinity determined here by direct methods contrasts strongly with the picomolar value previously determined using competition assays.[7] A similar difference was observed by Zambelli *et al*. between values obtained through direct and indirect methods with NikR from *H. pylori*.[11] It was argued that the competitor used in these assays, EGTA, may not be innocent: the presence of EGTA inhibits NikR binding to DNA[7a] suggesting that EGTA may form a ternary complex with NikR and Ni(II), which would affect the apparent dissociation constant of the Ni(II)–NikR complex. Furthermore, the value of the stability constant of the Ni–EGTA complex, which is fundamental for the calculation of the affinity of the HA site, is subject of disagreement spanning several orders of magnitude.[15] Finally, we should also mention that when relevant, we have also taken the secondary Ni(II) sites into account. These have only slightly poorer affinity for Ni(II) than the HA site, and obviously, when not accounted for, Ni(II) binding by these sites (*cf*. Fig. 2A, C and B, D) will lead to an overestimation of the affinity of NikR for Ni(II) in a competition assay. In fact, the possible presence of a small amount of residual Ni(II) in the LA sites in the filter binding assay, wrongly assumed to be bound to the HA site, may be the reason for the slightly higher affinity obtained calculated using this technique (82 nM) *versus* the other techniques applied in this work (~370 nM).

One can now question the claims that *E. coli* NikR has two distinct affinities for its cognate DNA,[7] based on whether Ni(II) is bound to the HA sites only, or also to additional site(s). The lower affinity for DNA binding was concluded from experiments performed with an equimolar mixture of Ni(II) and NikR, assuming that NikR was fully saturated with Ni(II). We know now that probably only a fraction of the NikR is saturated with Ni(II) under these conditions. The higher affinity for DNA observed in the presence of excess micromolar Ni(II) may thus simply correspond to conditions where the HA site is fully occupied.

In the cellular environment, Ni(II) is thought to be present in nM–μM concentrations. The major Ni(II) binding proteins have approximately μM affinity for their cofactor.[10] It is thus consistent that NikR, a major regulator of nickel levels in the cell, displays a sensitivity to Ni(II) at micromolar levels. Here we show that this is indeed the case, and that previous estimations of the affinity of NikR for Ni(II) are too high.

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Notes and references

‡ The *nikR* gene containing the Q2E mutation, described previously,[8] was used as a template for the H89N mutation. The resulting Q2E/H89N mutant (verified by sequencing) was expressed and purified as Q2E NikR, and Q2E and wt NikR were over-expressed and purified as before.[8] The Q2E mutation was originally introduced for cloning reasons and has no effect on Ni(II) binding, as confirmed in this and previous[8] studies. Protein concentrations are expressed in monomer units.

§ Ni(II) titrations were performed with Q2E NikR (between 0.6 and 860 μM), in 20 mM HEPES pH 8.0, 0.1 M NaCl, 293 K. The samples were allowed to equilibrate for >30 min. Depending on the NikR concentration, cuvettes were used with optical path lengths of 0.1, 1 and 10 cm.

¶ Filter binding assays were performed as described.[8,10f] Wt, Q2E and Q2E/H89N NikR were incubated for 45 min at room temperature with varying concentrations of $^{63}NiCl_2$, and subsequently immobilized on buffer-equilibrated 0.2 μm Sequiblot PVDF membranes (Biorad). The membranes were washed thoroughly with buffer. Protein-bound ^{63}Ni was quantified by counting the radioactivity of the filters. Controls were performed as described.[8] Background radioactivity (in the absence of protein) was negligible. In some experiments, the NikR variants were washed with a 250-fold excess of Na_2EDTA for 20 min before immobilization and washing, as above.

‖ Time-dependence of Ni(II) binding by Q2E NikR was followed at 302 nm, using a Bio-logic SFM400 stopped-flow apparatus equipped with a MOS-450 UV-vis spectrophotometer, in 20 mM HEPES pH 8.0, 0.1 M NaCl, 293 K. $NiSO_4$ (between 0.2 and 6.4 μM) was mixed with NikR (between 2 and 64 μM), in series of experiments with a Ni(II) : NikR ratio of 1 : 10 and 1 : 20. $NiSO_4$ was also mixed at a fixed concentration (6 μM), with NikR between 10 and 105 μM. NikR was consistently in excess to avoid protein precipitation. The average was taken of at least 5 traces for subsequent analysis.

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