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Potassium Is Critical for the Ni(II)-Responsive DNA-Binding Activity of *Escherichia coli* NikR

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Nickel ions are used by many organisms but intracellular concentrations must be controlled to minimize toxic effects on cellular metabolism.[1–3] This task often falls to metal-responsive transcription factors.[4,5] An example of such a metalloregulator is *Escherichia coli* NikR, which influences the uptake of nickel by repressing transcription of the *nik* operon encoding a nickel importer in response to nickel.[6,7]

NikR binds DNA as a tetramer (Figure S1),[8,9] with two functional domains connected by flexible linkers. The N-termini of two monomers intertwine to form a ribbon-helix-helix (RHH) DNA-binding domain, two of which flank the tetrameric center composed of four C-terminal nickel-binding domains.[8,10] NikR binds stoichiometric Ni(II) (4 ions per tetramer, holo-NikR) at the interface of the tetramer, which activates binding to a dyad symmetric DNA sequence that overlaps the *nik* transcription start site.[9,11] There is also evidence that excess Ni(II) induces a tighter NikR-DNA complex, with the K_D decreasing from nanomolar to picomolar,[9,11,12] and that this effect is a nickel-selective response.[9] This observation has led to the suggestion that one or more additional allosteric metal-binding site(s) exist on NikR, often referred to as the "low-affinity" Ni(II) site.[4,13]

The precise mechanism of metal-activated DNA binding is not yet known. The crystal structure of the *E. coli* Ni(II)-NikR-DNA complex reveals two potassium ions bridging the metal- and DNA-binding domains of the protein (Figure S1).[8] Mutagenesis of two of the potassium ligands, either Glu30 and/or Asp34, impairs NikR-DNA binding in vitro[8,14] and NikR function in vivo.[14] However, the identity of the metal ion that occupies this site is a subject of debate. There is speculation that the K^+ ions are placeholders for the low-affinity Ni(II) ions, primarily because Ni(II) is best modeled into the analogous sites in a *Pyrococcus horikoshii* NikR-PO_4^{3-} structure.[14] However, the O_6 coordination with long ($\geq$2.7 Å) bonds is more reasonable for K^+ than for Ni(II),[15,16] and molecular simulations with the NikR-DNA complex reveal an energetic preference for potassium at this location.[17] In an effort to further our understanding of the mechanism of action of this nickel-responsive regulator, we examined the functional role of K^+ in the DNA-binding activity of NikR.

All previous DNA-binding experiments with stoichiometric or excess nickel were performed in the presence of 100 mM KCl or NaCl.[9,12] When the larger monovalent cation Cs^+ (ionic radius of 1.67 Å versus 1.33 Å for K^+) was used in the DNase footprinting experiments, no binding was detected with up to 4 μM holo-NikR (Figure S2), a protein concentration well above the nanomolar K_D observed with millimolar K^+ (Table 1). Control experiments demonstrated that substituting K^+ with Cs^+ did not affect the 2° or 4° structures of NikR as reported by circular dichroism and size exclusion chromatography, respectively, or the high-affinity Ni(II)-binding properties as determined by electronic absorption spectroscopy and EGTA competition experiments (Figure S3 and data not shown). To confirm that it is the absence of K^+ that is responsible for the lack of observed DNA binding by NikR, K^+ was titrated back into the DNase footprinting samples containing holo-NikR in CsCl. DNA binding was restored and produced a footprint on the *nik* recognition sites at ~1 mM KCl (Figure 1B). Upon addition of excess Ni(II), protein precipitation complicated the analyses of the DNase footprinting experiments (data not shown). For this reason, NikR-DNA binding in CsCl and excess Ni(II) was examined by using mobility-shift assays (MSA) (Figure

Table 1. Apparent DNA-Binding Affinities of NikR Protein[a]

	WT/KCl	D34A/KCl	WT/CsCl	D34R/CsCl
1:1 Ni(II)	33 ± 6^b	Delocalized[b]	$>1000^d$	27 ± 1^b
Excess Ni(II)	0.05 ± 0.01^c	3 ± 1^b	$>1000^d$	0.10 ± 0.03^c

[a] Concentrations of protein at 50% saturation ($\times 10^{-9}$ M), the average of a minimum of two independent experiments ± SD. [b] Determined by DNase Footprinting. [c] Determined by MSA experiments performed with 35 μM Ni(II). [d] No shift or footprint was detected with up to 1 μM protein in CsCl.

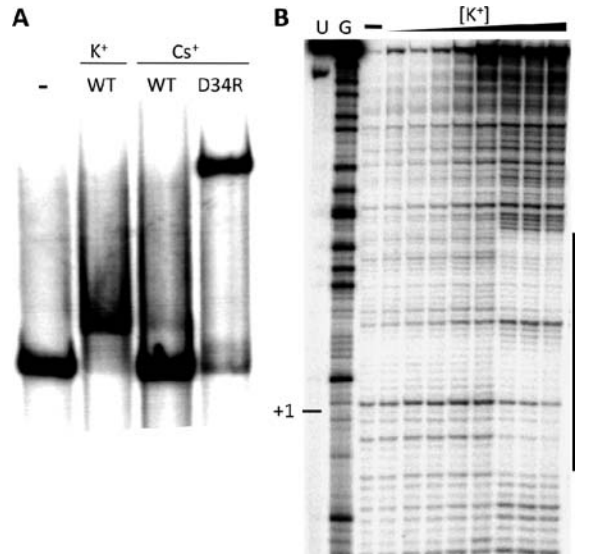

Figure 1. K^+ requirement for NikR DNA binding. (A) Ni(II)-NikR (1 μM, WT) in 100 mM KCl or CsCl and Ni(II)-D34R (1 μM) in 100 mM CsCl were incubated with DNA containing the *nik* recognition sequence. Reactions were analyzed on a 7% native gel with 35 μM $NiSO_4$ in the gel and running buffer. (B) KCl (5 nM to 100 mM) was titrated into 10 nM Ni(II)-NikR in CsCl and incubated with a 133-bp DNA probe for 1 h at room temperature prior to the addition of DNase I and analysis on an 8% denaturing polyacrylamide gel. The transcription start site (+1) and area of protection are indicated. G, G reaction; U, uncut DNA.

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1A).[9] Again, no DNA binding is observed with up to 1 μM NikR in the absence of K^+, but all of the DNA was bound if K^+ was included. These results demonstrate that K^+ is required for all NikR DNA-binding activity, whether in the presence of stoichiometric or excess nickel.

Mutating either Glu30 or Asp34 in NikR to alanine weakens the DNA affinity of NikR in MSAs performed with excess nickel by at least 3 orders of magnitude and results in a significantly slower mobility complex (Figure S4).[8,14] To analyze the effect of the D34A mutation on the DNA-binding response of NikR, DNase footprinting was performed. The protection afforded by holo-D34A is diffused along the DNA, in contrast to the distinct footprint produced by wild-type NikR (Figure S5), indicating a critical role for the K^+ site in sequence specific DNA binding. Furthermore, following incubation with 35 μM excess Ni(II) D34A produced a discrete footprint similar to that of wild-type NikR (Figure S5), with an apparent K_D for the D34A-DNA complex of 3 ± 1 nM (Table 1). This improvement in DNA binding suggests that the "low-affinity" Ni(II) ions bind at a location distinct from the K^+, a result consistent with the spectroscopic evidence for several histidine ligands on the additional Ni(II), residues that do not coordinate the potassium.[18] Furthermore, the observation that excess nickel restores the sequence-specific DNA binding disrupted by the D34A mutation suggests that the metal ions have overlapping roles.

In other Na^+- or K^+-dependent proteins, replacing a Glu or Asp ligand with a basic amino acid abolishes the Na^+ or K^+ requirement by structurally substituting for the cation.[19,20] To test the model that K^+ serves as a structural component required for DNA binding by NikR, the K^+ ligand Asp34 was mutated to an Arg (Figures S1 and S3). DNase footprinting experiments demonstrated that in the absence of K^+ holo-D34R produces a footprint with a similar location and affinity as that observed for wild-type Ni(II)-NikR in the presence of millimolar K^+ (Table 1 and Figure S6). Furthermore, the addition of excess nickel activates tighter DNA binding. This observation was confirmed by MSA experiments, in which half-maximal DNA binding by D34R in CsCl was observed at $(1.0 \pm 0.3) \times 10^{-10}$ M protein (Figure S4 and Table 1), a DNA-binding affinity that is only 2-fold weaker than that of wild-type NikR in the presence of K^+ and excess Ni(II) (Table 1). Although D34R replaces the functional requirement for K^+, the mutant exhibits a slower mobility complex in MSAs than wild-type NikR (Figures 1A and S4), the same discrete shift as produced by D34A (Figure S4). Nevertheless, mass spectrometry and MSA experiments confirmed that D34R binds DNA as a tetramer (Figures S7 and S8), as does wild-type NikR.[9] Thus the mechanism of K^+-dependent NikR-DNA binding cannot be recapitulated perfectly by a single residue substitution, as noted in other systems.[19,20]

The loss of DNA binding in the absence of potassium is dramatic. However, it is unlikely that a K^+-free form of NikR is physiologically relevant *in vivo* or that potassium has a regulatory role in the function of NikR.[21] Given that the intracellular concentration of K^+ in *E. coli* is >150 mM[22] and only 1 mM K^+ is needed for DNA binding, it is reasonable to assume that *in vivo* the K^+ site is constitutively filled in the holo-NikR-DNA complex. Glu30 and Asp34 are present in NikR orthologues, but the use of K^+ may not be conserved. For example, mutation of the corresponding residues in *Helicobacter pylori (H. pylori)* NikR had no measurable effect on DNA binding.[23] However, several divalent metals other than Ni(II) influence the activity of *H. pylori* NikR,[23–25] so perhaps NikR has adopted secondary metal-binding sites that function in distinct cellular environments.

The experiments described here were designed to assess the use of K^+ by the *E. coli* metalloregulator NikR. The results reveal that K^+ is a key component of the Ni(II)-responsive DNA-binding activity of this protein. Selective activation of enzymes by K^+ occurs in many instances, but to our knowledge this is the first example of a K^+-dependent transcription factor. A comparison of the structures of the holo-protein on its own and in a DNA complex reveals that the α2 helix is unwound in two of the monomers to allow the DNA-binding domains to rotate around to the same face of the repressor and bind to the two half-sites in the DNA recognition sequence (Figure S1).[8] Given the location of the potassium sites, it is reasonable to speculate that the cations reinforce the tertiary structure of NikR during this structural adjustment. These results, in combination with other biochemical and structural analysis, support the hypothesis that there are three distinct metal-binding sites that regulate Ni(II)-responsive DNA binding by NikR, the high- and low-affinity Ni(II) sites, and a K^+ site.[13]

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Supporting Information Available: Materials and methods, structure of the NikR-DNA complex (S1), DNase footprinting of holo-NikR with and without potassium (S2), MSA of D34R and D34A (S4), DNase footprinting of D34A and D34R (S5 and S6), characterization of the mutants 2° and 4° structures as well as nickel binding (S3), and MSA and MS experiments to determine the D34R-DNA stoichiometry (S7 and S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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