support the use of siRNAs to knock down splice variants, gene isoforms, and mutants bearing single nucleotide variations. Now, with this information in hand, RNAi may prove even more useful in functional genomic studies and in the treatment of human disease.

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Sensing Nickel: NikRs with Two Pockets

NikR represses expression of a nickel transporter in response to elevated levels of Ni(II). Recent results suggest that repression is elicited by binding of nickel to a high-affinity site, but a low-affinity binding pocket may also play a role.

Metal ions present a conundrum to the cell: they are essential for life, yet they can also be toxic when present in excess. In recent years, considerable progress has been made in defining the pathways by which cells acquire, distribute, store, and export metal ions. Metal ion homeostasis typically involves one or more high-affinity uptake pathways that are induced when metal ions are limiting and storage or efflux mechanisms induced when metals are in surplus. Responsibility for regulating these opposing pathways rests with metalloregulatory proteins that sense the intracellular levels of metal ions. In this issue of *Chemistry and Biology*, Chivers and Sauer report biochemical analyses of one such regulator, NikR, that has two distinct metal binding pockets: a high-affinity and a low-affinity site [1].

NikR is a member of the ribbon-helix-helix family of DNA binding proteins and represses Ni(II) transport when nickel levels in the cell are sufficient [2, 3]. Previously, Chivers and Sauer [4] attempted purification of NikR by loading the protein on a Ni-nitrilotriacetate (NTA) affinity column: a common expedient used for the affinity purification of proteins bearing histidine repeats (His-tagged proteins). Unexpectedly, NikR stripped Ni(II) away from NTA and therefore failed to bind the column. Their subsequent studies revealed two Ni(II) binding sites per NikR monomer: the high-affinity site detected in the metal-chelate chromatography and a second, much weaker site (an estimated $K_d > 10^{-5}$ M). Repressor with Ni(II) bound to both sites interacts with operator DNA with exceptionally high affinity (a K_d of 15 pM) [4].

In the current study, Chivers and Sauer [1] characterize the interaction of Ni(II) with the high-affinity site ($K_d \sim 7$ pM) in the carboxy-terminal domain of NikR, which also contains determinants for tetramer formation. Occupancy of the high-affinity Ni(II) sites is sufficient for operator binding ($K_d \sim 30$ nM). This is a reasonably high-affinity interaction for a gene-specific regulatory protein, but is 1000-fold weaker than the affinity exhibited when both Ni(II) sites are occupied. Since both forms of NikR bind operator DNA, albeit with differences in affinity and extent of binding, it is natural to wonder which form mediates repression of the nickel transport operon in vivo.

To address this question, the authors have measured the intracellular concentration of active NikR as ~125 tetramers per cell (200 nM). Since a single Ni(II) ion in the cytosol represents a concentration of ~1.6 nM, well above the dissociation constant of the high-affinity site, NikR will serve as a high-affinity sink for Ni(II). The au-

thors estimate that saturation of NikR with Ni(II) at the high-affinity sites, requiring ~500 Ni(II) ions per cell, would result in ~90% operator occupancy.

While these estimates are illuminating, some caveats are worth noting. First, binding of Ni(II) to the ~125 NikR tetramers in the cell will likely be distributive at low concentrations of Ni(II), and the resulting partially saturated tetramers may not bind operator DNA. Hence, it seems improbable that NikR could repress transcription in response to very low levels of Ni(II) per cell. Second, the affinity of NikR for DNA was estimated using DNase I footprinting of small fragments. In the cell, the concentration of free NikR could be significantly reduced due to the large excess of nonspecific DNA [5]. The magnitude of this correction is difficult to estimate, but in other systems it can be substantial. Third, the authors have measured NikR levels under anaerobic conditions: in aerobically growing cells, NikR levels are expected to be much lower [2] and it therefore seems unlikely that saturation of the high-affinity Ni(II) sites would lead to physiologically relevant repression. Even with the levels of NikR present in anaerobically grown cells, it is difficult to ascribe the >200-fold repression seen for the *nikA* operon [2] to a population of repressors that results in 90% operator occupancy. It is even conceivable, as the authors note, that NikR with only the high-affinity sites occupied may activate expression of the nickel uptake system, and binding of nickel to the lower-affinity sites may mediate repression. This is an attractive notion, since significant transcriptional induction can occur with only fractional operator occupancy, whereas efficient repression requires that the operator be nearly saturated with protein. Ultimately, deciphering the roles of the high- and low-affinity Ni(II) binding sites will require genetic analysis of mutations that eliminate one or the other site.

Although the functions of the two, very different metal binding pockets in NikR have not yet been elucidated, this work brings to the fore several questions engaging researchers in metalloregulation: how are metals stored within the cell? Are there specific metallochaperones for all the essential trace metals? Is there a kinetically labile pool of loosely chelated metals available for biosynthetic purposes? Current models posit that the cytoplasm has excess metal binding capacity [6, 7]. At equilibrium, one expects metals to first saturate the highest-affinity sites, such as those found in metalloenzymes, although incorporation may require cotranslational insertion or specific metallochaperones. As levels increase, metals interact with storage sites within proteins (such as ferritin or metallothionein), on low molecular weight carriers, or in subcellular compartments. At yet higher concentrations, metals will bind adventitiously to the myriad potential binding partners in the cell including small molecules, low molecular weight thiols, proteins, nucleic acids, and other anionic polymers. These latter, adventitiously bound metal ions can have toxic consequences by, for example, catalyzing hydroxyl radical formation [8, 9].

Metalloregulators must function at the crossroads: they bind their cognate metal ions less tightly than the functional high-affinity and storage sites but more tightly than the numerous adventitious sites that can lead to cell damage. Thus, the site in NikR that elicits repression of nickel transport must have an affinity appropriately poised to prevent the accumulation of Ni(II) to toxic levels. Is this the high- or the low-affinity binding pocket? In the case of the bacterial Zn(II)-sensing metalloregulators (Zur, ZntR, and SmtB), Zn(II) dissociation constants are in the pM range [6, 10], similar to the high-affinity site in NikR. Elegant studies of copper transport within yeast have demonstrated that there is no pool of free copper that can activate the metalloprotein superoxide dismutase (SOD) in the absence of a specific, copper binding chaperone [7]. The absence of "free" copper in the cell is due, at least in part, to the action of metallothioneins: in strains lacking metallothioneins, very high levels of copper can activate SOD [7]. Influenced by these observations, Chivers and Sauer seem to favor a role for the high-affinity Ni(II) site in mediating transcriptional repression [1].

While little is yet known regarding the details of nickel trafficking within *E. coli*, the two binding pockets of NikR undoubtedly hold important clues. Saturation of the high-affinity sites corresponds to an estimated nickel content of 500 atoms/cell and generates a form of the protein that is, the authors argue, sufficient for operator binding and repression. Binding of nickel to the lower-affinity sites will not reach significant levels until nickel concentrations approach the K_d of 30 μM. The dramatic effect of this second, low-affinity Ni(II) site on operator binding suggests biological relevance. Thus, the nickel content of the cell may reach quite high levels at least under some conditions.

Support for the idea that repression might be controlled by the lower-affinity Ni(II) site comes from studies of urease activation in the closely related bacterium, *Klebsiella aerogenes*. Activation of urease is assisted by UreE, a Ni(II) metallochaperone that binds Ni(II) ions with a K_d of ~10 μM [11]. Even in the absence of UreE, provision of cells with very high levels of nickel allows urease activation, albeit slowly [12]. This UreE-independent pathway for urease activation proceeds in vitro at Ni(II) concentrations above 100 μM [13], suggesting that such levels may also be achieved in vivo. Clearly, this would be more than sufficient to populate the lower-affinity site in NikR [4].

The extent to which other metal ions are chaperoned in the cell is not yet clear [14]. Work in bacterial systems argues in favor of kinetically labile pools of metal ions which, while not chemically "free," function as a storage reservoir. In *E. coli*, for example, there is a pool of loosely held ferrous iron that can be monitored by treatment with a cell-permeable chelator and electron paramagnetic resonance [9]. Mutation of the major regulator of intracellular iron homeostasis, *fur*, leads to an increase in this labile iron pool [9]. *B. subtilis* responds to oxidative stress by inducing uptake of Zn(II), a nonredox active metal thought to displace copper, iron, and other redox active metals from adventitious sites that could oxidatively damage proteins and nucleic acids [15]. DNA microarray studies in *B. subtilis* also provide glimpses of these pools by monitoring global patterns of metalloregulation. For example, an influx of Mn(II) represses the Fur regulon. Contrary to expectation, this is not due to a direct interaction with Fur, but rather reflects the

displacement of Fe(II) from a labile pool by incoming Mn(II). Similarly, Zn(II) influx leads to a transient repression of both the Mn(II)-responsive MntR regulon and the Fe(II)-responsive Fur regulon. Thus, the emerging picture is one of labile pools of metal ions that can rapidly exchange when metal homeostasis is perturbed. If it is, in fact, the low-affinity Ni(II) site that regulates uptake, the pools of Ni(II) ions in *E. coli* may also be substantial.

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