Helicobacter pylori NikR Protein Exhibits Distinct Conformations When Bound to Different Promoters*

Received for publication, October 19, 2010, and in revised form, March 10, 2011. Published, JBC Papers in Press, March 10, 2011, DOI 10.1074/jbc.M110.196055

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Helicobacter pylori NikR (HpNikR) is a ribbon-helix-helix (RHH) DNA-binding protein that binds to several different promoter regions. The binding site sequences are not absolutely conserved. The ability of HpNikR to discriminate specific DNA sites resides partly in its nine-amino acid N-terminal arm. Previously, indirect evidence indicated that the arm exists in different conformations when HpNikR is bound to the nixA and ureA promoters. Here, we directly examined HpNikR conformation when it was bound to nixA and ureA DNA fragments by tethering (S)-1-[bis(carboxymethyl)amino]methyl)-2-[4-[(2-bromoacetyl)amino]phenylethyl]-(carboxymethyl)amino)acetic acid, (Fe-BABE, (S)-[bis(carboxymethyl)amino]methyl)-2-[4-[(2-bromoacetyl)amino]phenylethyl]-(carboxymethyl)amino)acetic acid, iron(III) to different positions in the N-terminal arm and RHH DNA binding domain. Different cleavage patterns at each promoter directly demonstrated that both the RHH domain and the arm adopt different conformations on the nixA and ureA promoters. Additionally, the two RHH domain dimers of the HpNikR tetramer are in distinct conformations at ureA. Site-directed mutagenesis identified an interchain salt bridge (Lys48-Glu47) in the RHH domain remote from the DNA binding interface that is required for high affinity binding to ureA but not nixA. Finally, DNA affinity measurements of wild-type HpNikR and a salt bridge mutant (K48A) to hybrid nixA-ureA promoters demonstrated that inverted repeat half-sites, spacers, and flanking DNA are all required for sequence-specific DNA binding by HpNikR. Notably, the spacer region made the largest contribution to DNA affinity. HpNikR exhibits a substantially expanded regulon compared with other NikR proteins. The results presented here provide a molecular basis for understanding regulatory network expansion by NikR as well as other prokaryotic regulatory proteins.

*This work was supported by National Science Foundation Grant MCB 0520877.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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3 The abbreviations used are: HpNikR, H. pylori NikR; EcNikR, E. coli NikR; RHH, ribbon-helix-helix; Fe-BABE, (S)-1-[bis(carboxymethyl)amino]methyl)-2-[4-[(2-bromoacetyl)amino]phenylethyl]-(carboxymethyl)amino)acetic acid, iron(III).
The EcNikR-operator structure revealed DNA interactions similar to those of the Arc and MetJ repressors (27). Additionally, the nickel binding C-terminal domain interacts with the phosphate backbone of the central base pairs that separate the inverted repeat sequences in the two promoter regions. These differences exist beyond the N-terminal arm when the protein is bound to the DNA, so any differences between the protein-DNA binding affinity (5). Here, we have further examined this conformational difference using chemically modified and new site-directed mutants of HpNikR, in combination with engineered promoter sequences, to demonstrate that protein conformational differences exist beyond the N-terminal arm when the protein is bound to the DNA regions-flanking the inverted repeat sequences in the two promoter regions. These observations will help to understand NikR regulon expansion in H. pylori and, more generally, the interplay between protein and DNA sequence in prokaryotic regulon expansion.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis of HpNikR—Site-directed mutagenesis of HpNikR was carried out using the QuiKChange protocol (Stratagene, La Jolla, CA). Complementary oligonucleotides (Table 1) with the mutated codon were used with plasmid pEB116 (5) to generate C96A (pEB173) and each salt bridge mutant. The**
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T3C, N5C, and N20C mutants were generated using pEB173 as a template. The presence of each mutation was verified by DNA sequencing (SeqWright, Houston, TX).

Protein Expression and Purification—All HpNikR proteins and mutant variants were expressed and purified as described previously (5). The T3C, N5C, and N20C mutant proteins were purified with 1 mM β-mercaptoethanol present throughout the purification protocol. Protein concentration was determined in 6 M guanidine hydrochloride using ε_{270} = 9895 M⁻¹ cm⁻¹ (31).

Promoter Fragments; Cloning and Labeling—Hybrid nixA-ureA promoter fragments were generated by two sequential rounds of PCR using overlap extension with two oligonucleotides that contained the base mutations and flanking primers designed to amplify ∼200–400 bp of DNA spanning the nixA or ureA promoters and genes (Table 1). The final PCR product was digested with EcoRI (New England Biolabs, Beverly, MA) and KpnI (New England Biolabs) and ligated into pBluescript (Stratagene). DNA sequences of the hybrid promoters were verified by sequencing (SeqWright).

Promoter fragments for the Cys mutant DNase I footprinting and (5) 1-[bis(carboxymethyl)amino]methyl]-2-[4-[2-bromoacetamido]phenylthioy]carboxymethyl)amino]acetic acid, iron(III) (Fe-BABE) (Dojindo Laboratories, Kumamoto, Japan) cleavage assays were generated by end-filling EagI-digested, gel-purified PCR products (primers EB628 and EB618 for nixA and EB631 and EB620 for ureA; Table 1). End-fill reactions used 0.2 μM PCR product, 1.0 μl of 3′-5′ exonuclease (New England Biolabs), and [α-32P]dGTP (PerkinElmer Life Sciences) in a total volume of 40 μl. Excess [α-32P]dGTP was removed using a nucleotide exchange kit (Qiagen, Valencia, CA). Wild-type and hybrid promoter fragments used in the mobility shift assays were generated as follows. 0.5 μM forward (5′) primer (EB629 for nixA and EB632 for ureA; Table 1) was 5′-end-labeled with [γ-32P]ATP (PerkinElmer Life Sciences) and 1.0 μl of T4 polynucleotide kinase (New England Biolabs) in a total volume of 40 μl. Excess [γ-32P]ATP was removed by desalting, and the purified primers were used in a PCR with the corresponding reverse primers (EB618 for nixA and EB620 for ureA; Table 1) using plasmid DNA as the template. The resulting labeled fragments were purified using a Qiagen PCR purification kit (Qiagen).

Fe-BABE Modification and DNA Cleavage by Modified Cys Mutants—Purified HpNikR Cys mutants were desalted into 20 mM Tris-Cl (pH 8.0), 300 mM NaCl to remove the β-mercaptoethanol, and NiCl₂ was added back to approtein at a stoichiometry of 2:1 Ni²⁺/HpNikR monomer to ensure saturation of the high affinity binding site. 40 μM protein was incubated with 15 mM Fe-BABE in a final reaction volume of 37 μl and incubated at 37 °C for 1 h according to established protocols (32). The reaction was quenched with an equal volume of 1 M Tris-Cl (pH 8.0) and desalted two times into 20 mM Tris-Cl (pH 8.0), 300 mM NaCl to eliminate unreacted Fe-BABE.

Fe-BABE cleavage reactions were performed in the same buffer used for DNase I footprinting and used a variation of a previously published protocol (32). The reactions were started with 5 μl of a 10× stock of freshly mixed sodium ascorbate (100 mM; stored in aliquots at −20 °C) and H₂O₂ (250 mM), incubated for 2 min at 22 °C, and quenched with 36 μl of a 2.5× stock solution of thiourea (100 mM) and EDTA (75 mM). DNA fragments were analyzed by denaturing polyacrylamide gel electrophoresis.

DNase I Footprinting—DNase I footprinting was performed as described previously (13, 33) in a binding buffer containing 10 mM Tris-Cl (pH 8.0), 100 mM KCl, 3 mM MgCl₂, and 50 μM NiCl₂. Labeled DNA fragments were incubated with protein at 22 °C for 30 min prior to the addition of DNase I (Sigma) (final concentration 300 ng/ml). Formic acid cleavage of labeled DNA was performed using the standard protocol for Maxam-Gilbert sequencing (2). DNA fragments were analyzed by denaturing gel electrophoresis.

Electrophoretic Mobility Shift Assays—Mobility shift assays were performed using 7% polyacrylamide gels and electrophoresis buffer containing 50 mM Tris (pH 8.8), 25 mM boric acid and with 50 μM NiCl₂. The binding buffer was identical to that used for DNase I footprinting except that 10 μg/ml E. coli thioredoxin (34) and 4 ng/μl salmon sperm DNA (Fisher) were added to prevent nonspecific HpNikR accumulation in the gel wells. Labeled DNA fragments were incubated with HpNikR or mutant proteins at 22 °C for 30 min, and 20 μl of the 25-μl total volume was loaded directly onto a running gel (120 V).

Apparent affinities measured by mobility shift and DNase I footprinting assays were calculated from binding curves determined by the ratio of bound (all shifted species) versus free counts as quantified using a GE Healthcare Typhoon Trio variable mode imager and ImageQuant version 5.1 software. The data were fit using MICROMATH SCIENTIST version 2.01 and the following equation,

\[ y = 1/(1 + (K_d/x)^n) \]  
(Eq. 1)

where \( y \) represents fraction of DNA bound (ratios described above), \( K_d \) is protein concentration required for half-maximal DNA binding, \( x \) is protein concentration, and \( n \) is the Hill coefficient. All reported affinities are the average of at least two independent experiments using a dilution series of 16 protein concentrations, and the S.D. value is also reported.

Circular Dichroism and Fluorescence Spectroscopy—CD spectra were collected on a Jasco J-715 spectropolarimeter using a 900-μl sample volume in a cylindrical cuvette with a 1-cm path length. All spectra were collected at 22 °C in a buffer containing 20 mM Tris-Cl (pH 7.5), 100 mM NaCl.

Urea melts were performed with 6 μM protein that was incubated in the absence or presence of increasing concentrations of urea in 20 mM Tris-Cl (pH 7.5) 100 mM NaCl at 22 °C for a minimum of 16 h prior to measurements. Fluorescence spectra were collected on a Cary Eclipse fluorescence spectrophotometer in a 150-μl, 1-cm path length Hellmann quartz cuvette. Samples were excited at 295, and emission spectra were collected from 315 to 380 nm with excitation and emission slit widths set to 10 nm. Center of mass calculations were performed as described previously for the Arc repressor (35).

RESULTS

To test if HpNikR adopts distinct conformations when bound to DNA, we endowed the protein with nuclease activity by covalent modification with Fe-BABE. This approach was...
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Cleavage of nixA—Fe-BABE-modified T3C or N5C HpNikR* also produced eight distinct cleavage regions on nixA that spanned the DNase I footprint (Fig. 2, a and c). Three regions were located outside (aOC1 to -3, for arm outer cleavage) and five inside (aIC1 to -5, for arm inner cleavage) of the inverted repeat half-sites, with all but one pair of the eight regions being separated by 2 bases and symmetrically positioned relative to the footprint. The cleavage observed for T3C and N5C often overlapped with that seen for N20C (Fig. 2, compare a, c, and e), with an offset of 1–2 bases/region. This suggests that the Fe-BABE moieties of Thr3, Asn5, and Asn20 are similarly but not identically positioned in space relative to the DNA backbone.

Cleavage of ureA—Fe-BABE-modified T3C or N5C HpNikR* cleaved ureA at six regions (Fig. 2, d and f) that can be grouped as three outer regions (aOC1 to -3) and three inner regions (aIC1 to -3). aIC1 and aIC2 were distinct from all other HpNikR cleavages because of their size (6 bases/region), spanning much of the DNA spacer between the two inverted repeat half-sites. Notably, there was larger spacing between the upstream cleavage regions compared with the downstream regions (aOC1-aOC2 versus aIC3-aOC3; aOC2-aIC1 versus aIC2-aIC3), resulting in an overall asymmetric cleavage pattern for ureA. This cleavage asymmetry was similar to that observed for N20C cleavage of ureA and is consistent with the two arms and one RHH domain dimer from each half of the HpNikR tetramer.
being in two different conformations when bound to the \textit{ureA} promoter region. This result contrasts with the symmetric cleavage patterns observed for the three modified proteins when bound to \textit{nixA} promoter DNA. The distinguishable cleavage patterns observed for \textit{nixA} and \textit{ureA} with modifications of either the /H9251 helix or the arm provide strong support for different HpNikR conformations when bound to the promoter regions.

\textbf{Mutation of an RHH Residue That Does Not Contact DNA Selectively Impairs \textit{ureA} Binding}

The RHH domain of HpNikR is less stable to chemical denaturation than that of EcNikR (39). This reduced stability may correlate with conformational flexibility and expanded DNA sequence recognition. To probe residues that might contribute to this differing stability, we generated a K48A HpNikR mutant. This residue position is poorly conserved across NikR orthologs (39) and is often a small residue in RHH proteins. The K48A mutant was not affected in stability (supplemental Fig. S1) but showed profound differences in DNA binding affinity between the \textit{nixA} and \textit{ureA} promoters (Fig. 3 and Table 2). Based on examinations of different RHH-DNA co-complex structures, it is very unlikely that Lys48 of HpNikR directly contacts DNA.

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\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{The RHH domain and the N-terminal arm of HpNikR have different orientations when bound to the \textit{nixA} and \textit{ureA} promoters. DNase I protection is indicated at the left of each panel (a--d) with a solid black bar. Fe-BABE cleavage by each modified HpNikR is indicated at the right of each panel with multiple black (stronger) and gray (weaker) bars. e and f show the \textit{nixA} and \textit{ureA} promoters with cleavage patterns mapped onto the sequence. Cleavage sites for N20C-modified HpNikR are indicated by rOC or rIC, and cleavage sites for T3C- and N5C-modified HpNikRs are indicated by aOC or aIC. The predicted half-site sequences are highlighted in gray boxes (5). The DNase I footprints are indicated by square brackets. The numbering refers to the sequence positions relative to the start of transcription determined for each promoter (9, 56).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{An interchain salt bridge between Glu47 and Lys48 of the HpNikR RHH domain is required for high affinity binding to the \textit{ureA} promoter. Shown are electrophoretic mobility shifts of HpNikR K48A with \textit{nixA} (a) or \textit{ureA} (b) promoter fragments. Protein was serially diluted 1.7-fold from 500 nM to 175 pM. The \textit{leftmost lane} in each titration shows DNA alone. F, free DNA. B, bound DNA. Each titration was run on two gels, which are separated by vertical black lines.}
\end{figure}
differences beyond the RHH domain-DNA interface when the protein is bound to different promoter sequences.

**Lys^{48} Participates in a Salt Bridge That Is Required for High Affinity ureA Binding**

To better understand the role of Lys^{48} in HpNikR-DNA interactions, we examined different HpNikR crystal structures (40). The negatively charged side chains of two residues, Glu^{47} from the other polypeptide chain in the RHH dimer and Asp^{52} from the same chain (i to i + 4), are both close to Lys^{48} (Å3.8 Å). To determine if either residue interacts with Lys^{48} when HpNikR is bound to ureA, E47A and D52A mutants were constructed and tested for DNA binding in mobility shift assays. Both mutants bound to the nixA and ureA DNA fragments with only slightly weakened affinities (Table 2) compared with the 60-fold reduction of K48A affinity for ureA. The E47A and D52A mutants had 2- and 4-fold decreases in binding affinity for nixA, whereas both mutants showed a similar (6–7-fold) decrease in affinity for the ureA promoter region.

The absence of a significant decrease in affinity for the two mutants suggests that neither residue interacts with Lys^{48}, nor are they important by themselves for DNA binding. Alternatively, the remaining acidic side chain may partially compensate for the mutated residue and preserve the interaction with Lys^{48}. This possibility was explored with the E47A/D52A double mutant, which had lower affinity for the single mutants (12- and 37-fold, respectively; compare Table 2). The greater reduction in affinity for binding to ureA is similar to the effect of the K48A mutation. These data support the idea that at least one negatively charged residue interacts with Lys^{48} when HpNikR is bound to ureA, although the double mutant is not as impaired as K48A.

**Inverting Candidate Salt Bridge Residues Identifies a Critical Glu^{47}–Lys^{48} Interaction**

As a further probe of potential Lys^{48} interactions, two residue pair swap mutants were constructed (E47K/K48E and K48D/D52K), along with two single mutants (E47K and K48E). Intriguingly, the E47K/K48E double mutant displayed only minor decreases in affinity for nixA and ureA of 1.5- and 2-fold, respectively (Table 2). In contrast, the K48D/D52K mutant was severely impaired for DNA binding to both promoter fragments (164-fold lower affinity for nixA and no detectable binding for ureA up to 5 μM protein). The similar behavior of the K48A and K48D/D52K mutants suggests that the double mutant cannot make the Lys^{48}-dependent interaction required for ureA binding. The E47K and K48E single mutants had reduced affinity for ureA (E47K) or both nixA and ureA (K48E) compared with the E47K/K48E charge swap double mutant.

These data are most consistent with the formation of a specific intersubunit interaction between the Glu^{47} and Lys^{48} side chains when HpNikR is bound to ureA. The various deleterious effects of the single mutants and the K48D/D52K on DNA binding affinity are also consistent with an important structural interaction involving Lys^{48}.

**The Effects of RHH Domain Mutations on DNA Binding Do Not Correlate with Protein Stability**

Urea melts were performed in the presence of stoichiometric Ni^{2+} to determine if the charge mutations affect DNA binding affinity by perturbing RHH domain stability. Domain unfolding was monitored by following the red shift and quenching of Trp^{54} fluorescence upon unfolding (41). Trp^{54} is in the linker between the RHH and C-terminal domains of HpNikR. Its fluorescence reports solely on RHH domain folding because comparison of fluorescence shift (as determined by center of mass calculations for each spectrum; see “Experimental Procedures”) with loss of CD signal at 230 nm from HpNikR urea melts indicates only a single fluorescence transition that coincides with the RHH domain transition in the CD data (supplemental Fig. S1).

The Trp fluorescence of several of the charge mutants was perturbed in the native protein prior to the addition of urea (supplemental Fig. S2). Given the spatial proximity of Trp^{54} to the positions of α2 helix mutations, these changes probably represent local perturbations of structure that influence the quenching of Trp fluorescence. Most of the charge mutants showed little to no change in the RHH domain unfolding transition (supplemental Fig. S1) with two exceptions. The K48D/D52K double mutant displayed an obvious loss in the cooperativity of unfolding. This decreased stability may explain the poor DNA binding affinities of this mutant (Table 2). The K48E mutant displayed reduced stability with a shift in the midpoint of the unfolding curve to significantly lower urea concentrations. This decrease in stability was almost fully alleviated in the E47K/K48E double mutant. These data indicate that the ability of HpNikR variants to bind nixA and ureA did not correlate with changes in RHH domain stability except for the destabilized K48D/D52K mutant. In this case, the severe disruption in DNA binding is probably the result of a more general structural perturbation rather than a specific salt bridge disruption. The absence of a correlation between protein stability and DNA binding affinity among the other mutants is consistent with a specific requirement for a Glu^{47}–Lys^{48} salt bridge.
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Half-sites, Spacers, and Flanking DNA Sequences Contribute to HpNikR High Affinity Binding to nixA and ureA

The stark difference in affinities of K48A HpNikR for the nixA and ureA DNA fragments lead us to probe the relative contributions of different DNA sequence elements to the affinity change. Typically, the inverted repeat sequences present in RHH DNA recognition sites are thought to provide the majority of the interactions necessary for specific protein-DNA complex formation (16, 23), although this has not been probed extensively. Thus, changes in half-site sequences represent likely candidates for influencing the conformation of HpNikR when bound to the nixA and ureA promoters. Comparison of the inverted repeats in the nixA and ureA promoter fragments, which are based on DNase I, Fe-EDTA, and DMS footprinting (5), indicated that 4 of the 12 bp of the nixA and ureA 6-bp half-sites are different (Figs. 2 and 4).

To identify the DNA sequence features that foster a “nixA-like” conformation of K48A HpNikR, several nixA-ureA hybrid promoters were constructed in which the two half-sites, the flanking sequences, and the intervening spacer regions of the two promoters were arranged in different combinations (Fig. 4). These constructs represent all of the possible transitions between the nixA and ureA promoter sequences, or vice versa.

Wild-type HpNikR exhibited only modest affinity reductions for all six of the hybrid promoters (~4–6-fold; Fig. 4). Substitution of the nixA or ureA half-sites in the context of the other promoter (ureA-HS and ureA-FL+SP in Fig. 4) had similar effects on affinity (3–4-fold decrease). Substitution of the spacer or flanking regions in either context had a slightly greater effect (5–6-fold). The limited decrease in affinity with the hybrid promoters indicated that the sequence changes did not substantially affect any key protein-DNA contacts. Nonetheless, the affinity changes in the hybrid DNA fragments suggest some conformational distortion of native HpNikR along the transition between native binding site sequences. However, the effects on affinity were neither additive nor synergistic.

In contrast to wild-type HpNikR, the K48A mutant was very sensitive to any substitution that increased the ureA sequence content of the promoter (Fig. 4), showing 9- and 10-fold decreases in affinity when the ureA half-sites (ureA-HS) or flanking regions (ureA-FL) were inserted into the nixA sequence. Surprisingly, the largest -fold decrease (59-fold) in response to exchange of a single sequence element occurred upon swapping the ureA spacer into the nixA sequence. A single sequence swap of this type was previously shown not to affect the EcNikR-DNA interaction (14). These data suggest that the ureA spacer destabilizes K48A binding more than either the ureA half-sites or flanking sequences. Similar to the results for wild-type HpNikR, these effects were neither additive nor synergistic. In particular, the ureA spacer was the main determinant of binding affinity when at least one nixA promoter element remained.

The affinity changes observed for the native and mutant HpNikR proteins support two key aspects of HpNikR DNA recognition: (i) sequence elements throughout individual promoter sequences are important for DNA binding, and (ii) protein conformational flexibility is required for HpNikR to recognize the two promoters. This flexibility relies at least in part on interactions involving Lys^48 in the context of binding to ureA.

**DISCUSSION**

The HpNikR regulon is more extensive than those of most RHH proteins (42), which generally bind to only one recognition site in their respective genomes. Here, three complementary lines of evidence indicate that the expansion of the HpNikR regulon includes differential recognition of various promoter regions through changes in protein-DNA interactions that affect protein conformation. HpNikR appears to have at least two conformations when bound to DNA, and protein conformation is sensitive to DNA sequence or structure beyond the inverted repeat sequences in the binding sites tested here. These results suggest that interpretation of HpNikR-DNA interactions in the context of the HpNikR regulon and nickel-dependent gene regulation in H. pylori should extend beyond previously inferred changes in DNA contacts afforded by the flexible N-terminal arm. The different protein conformations could be differentially sensitive to changes in intracellular phys-
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The dramatic reduction in HpNikR K48A affinity after substitution of the nixA spacer with that of ureA may help to compensate for the loss of the N-cap residue DNA contact and provide flexibility in DNA recognition.

The Fe-BABE cleavage data are consistent with DNA interactions outside of the inverted repeat sequences. Such interactions have been observed in other RHH-DNA complexes, both experimentally and structurally (23–25, 47). The hybrid promoter fragments provide complementary evidence in support of these interactions as well as differences between HpNikR-DNA interactions with ureA and nixA. Although the affinity changes of wild-type HpNikR for different promoters was modest, interactions of the K48A mutant revealed highly sensitive responses to subsets of the ureA promoter sequence swapped into the nixA fragment. These data show that a combination of half-site, spacer, and flanking sequences is required for high affinity DNA binding. Together with the evidence that the N-terminal arm impacts DNA binding specificity, this suggests that the structure of the DNA recognition sites outside of the HpNikR half-sites may be critical for specific DNA binding.

The role of the spacer DNA sequence in HpNikR DNA recognition is reminiscent of DNA binding by the 434 repressor protein (50). The central four bases of a 14-bp recognition motif are critical for 434 repressor binding to six distinct operator sites and, interestingly, are not all contacted by the repressor protein. DNA affinity is intimately linked to the A-T content of this 4-bp spacer, presumably due to the extent to which the spacer can overwind, compress, and properly orient the repressor binding.

These protein-DNA contacts are thought to help anchor the β-sheet motif to DNA (23). The structural mechanism by which Ni2+ binding controls NikR DNA binding is not yet fully understood but probably involves an allosteric network of residues that affects non-covalent interactions at the N- and C-domain interfaces (39, 48). The Lys48-Glu47 interaction may provide a connection between Ni2+ and DNA binding, as can be observed in one HpNikR structure (Protein Data Bank entry 2WVE) where a Lys48-Glu47-Lys137 linkage is present. However, this structure probably does not represent the ureA-bound conformation of HpNikR because the K48E/E47K swap mutant is unlikely to interact with Lys137 but is still competent for DNA binding.

There is also a key difference between the α2 helix N-terminal residues of HpNikR and EcNikR. This position is considered an N-cap residue, and makes a side chain to main chain hydrogen bond interaction (i to i + 4) that is known to stabilize the N terminus of an α-helix (49). This residue is Ser in HpNikR and Asn in EcNikR. In EcNikR, the Asn side chain also contacts the DNA phosphate backbone due to bifurcation of the polar amide group. This type of nonspecific DNA interaction seems less likely to occur in DNA-bound HpNikR because of the more limited hydrogen bond capability of the Ser-OH side chain, which probably also forms the N-cap hydrogen bond. Alternatively, if the Ser residue can interact with DNA, its position may be influenced by the relative orientation of helix α2 in a way that depends upon the interaction of Lys48-Glu47. DNA contacts made by the N-terminal arm of HpNikR may help to compensate for the loss of the N-cap residue DNA contact and provide flexibility in DNA recognition.

The effect of the K48A mutation on the HpNikR-ureA interaction was unexpected. The identification of a putative Glu47-Lys48 salt bridge between the α2 helices of the RHH dimer as a requirement for high affinity binding to ureA but not nixA adds a new perspective for the identification and understanding of differences in protein conformation that exist between HpNikR tetramers bound to different promoters. In particular, these data suggest that conformational changes are not restricted to the protein-DNA interface. The available crystal structures of HpNikR reveal that the Lys48-Glu47 interaction (Lys-Ne to Glu-Oδ or -Oε distance <3.2 Å) is present in several instances (38, 40). In many cases, only one of the two Lys residues in the RHH domain is visible. None of these structures includes DNA, although SO42− ions from the crystallization buffer are visible in some cases. There is no correlation between the occurrence of the salt bridge interaction and the presence of nickel ions in the structure. A Lys48-Asp52 interaction (<3.2 Å) can be seen in two of the structures (40), and although intrahelical salt bridges (i to i + 4) are known to influence helical stability based on model peptide studies (45, 46), this type of interaction does not appear to be important for the HpNikR-DNA interactions studied here.

Only a few NikR sequences have the Glu47-Lys48 residue pair (39), so this interaction does not appear to be widespread among NikR orthologs. It is possible that different residue pairs are used in a similar fashion by other NikR orthologs. Alternatively, residue 48 may interact with a different partner, such as residue 52, in some NikR proteins. The low sequence conservation of this protein region and its proximity to the interface of the Ni2+- and DNA-binding domains suggest that it may be particularly amenable to changes that influence function.

The location of the Glu47 and Lys48 in the α2 helix suggests that their interaction may affect the HpNikR DNA-contacting β-sheet differently at nixA and ureA. In every RHH protein-DNA co-crystal structure, there are interactions between residues at the N terminus of the α2 helix and the phosphate backbone flanking each end of the repeat sequences (23–25, 47).

These data are consistent with previous findings that showed a cation requirement for the arm truncation mutant nt9-HpNikR only for the ureA promoter in mobility shift assays (5). More detailed studies are required to understand the structural basis for these differences; however, it is likely that the N-terminal arm makes DNA contacts that are important for DNA binding. The two most likely possibilities are an encircling of the DNA by HpNikR, as has been demonstrated for λ repressor (44), and/or accommodation of spacer sequence asymmetry by altering DNA contacts via arm flexibility. We note that previous DMS footprinting results were identical for native and nt9-HpNikR and did not show protection of the few G residues in the spacer regions of either DNA fragment.

The role of the Glu47 and Lys48 in the structure of this protein region and its proximity to the interface of the α2 helix (49) are known to influence helical stability based on the structural mechanism by which the α2 helix differently at nixA and ureA. In every RHH protein-DNA co-crystal structure, there are interactions between residues at the N terminus of the α2 helix and the phosphate backbone flanking each end of the repeat sequences (23–25, 47).

The dramatic reduction in HpNikR K48A affinity after substitution of the nixA spacer with that of ureA supports the idea that their interaction may affect the HpNikR DNA-contacting β-sheet differently at nixA and ureA. In every RHH protein-DNA co-crystal structure, there are interactions between residues at the N terminus of the α2 helix and the phosphate backbone flanking each end of the repeat sequences (23–25, 47).

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that the spacer structure is an integral determinant of HpNikR DNA binding. Spacers from the eight promoters to which HpNikR directly binds exhibit highly variable A-T content, with no two spacers containing the same length runs of A. One possibility is that distinct HpNikR conformations recognize variable half-site spacing, which result from differences in spacer sequence content. In this case, the spacer would constitute a significant determinant of DNA binding affinity, as was observed for the nixA and ureA spacers. A half-site sequence may bind preferentially to a particular HpNikR conformation that enables stable complex formation. Stepwise selectivity via cooperative protein interactions after half-site binding has been observed for the RHH Mnt repressor binding to DNA (51).

The NikR tetramer binds to the largest operator site of any RHH protein. The greater spacing between half-sites is a consequence of the C-terminal nickel-binding domain, which is physically interposed between the RHH domains in the DNA complex. The DNA duplexes of many of the RHH-DNA co-crystal structures are bent to varying degrees (Arc, 50° (25); MetJ, 50° (24); CopG, 60° (29); FitA, 44° (52); EcNikR, 22° (27); ParR, 46° (53)), which further supports the idea that RHH proteins with different β-sheet motifs require variations in flanking DNA structure for optimal DNA binding. It is likely, then, that models of RHH protein-DNA interactions need to be expanded to include the analysis of DNA structural specificity outside of the minimal binding sites often predicted from lower resolution experiments.

HpNikR directly binds to at least eight different promoters (4–10, 12, 54), although detailed mapping of HpNikR-DNA interactions has only been performed for two of these (5). The interaction of HpNikR at the ureA promoter has been interpreted as transcriptional activation because of the enhancement in ureA expression upon the addition of nickel, which requires a functional nikR gene. However, transcriptional activation has not been demonstrated in vitro, so the simple conclusion that HpNikR-DNA interactions at nixA and ureA reflect different functional roles is probably premature. Preliminary binding experiments with K48A HpNikR and the HpNikR-repressed frpB4 promoter showed a ~50-fold reduction in affinity. Because the sequences of the half-sites and spacer of frpB4 are different from both nixA and ureA, this result suggests that HpNikR does not adopt conformations that are specific to the binding site position relative to the start of transcription but instead adapts conformation to sequence. Future studies that take a comprehensive approach to defining the molecular details of HpNikR-DNA interactions with several promoters will help to identify how flexibility and specificity in RHH domain-DNA interactions are achieved. The K48A mutant will be a useful tool for these studies.

Acknowledgments—We thank Jeff Iwig for technical advice and Jeff Iwig and Mike Bradley for helpful discussions.

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